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Visual system development in *Platynereis dumerilii*: insight from genetic engineering aproach

Vývoj vizuálního systému u *Platynereis dumerilii*: náhled pomocí metod genového inženýrství

DIPLOMA THESIS

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Ivana Dobiášovská

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ABSTRACT

Gene regulatory networks, underlying the molecular regulation of eye development are conserved across many animal phyla. Genes from the *Pax* family of transcription factors are one of the most conserved members through the evolution, regulating the development of crucial parts of eye, including the photoreceptor cells. *Pax* transcription factors are considered to be regulators of opsins, molecules providing the conversion of the light stimulus into the electrochemical signalisation in the photoreceptors cells. In this thesis, *pax6* and *pax2/5/8* transcription factors are investigated as potential regulators of eye development in *Platynereis dumerilii.*

pax6 and *pax2/5/8* transcription factors are tested as potential regulators of the *r-opsin* in *Platynereis*, based on the observed early expression onsets of these genes. Wild-type expression analysis of *pax6* and *pax2/5/8* using the whole mount RNA *in-situ* hybridization is provided, accompanied by the initial analysis of the *Platynereis pax6* knockout line. *pax6* heterozygote mutants are shown to be viable and able to reproduce, however, homozygote mutation of *pax6* in *Platynereis* is lethal. Our data suggest that transcription factors *pax2/5/8*, *otx* and *six3* are not regulated by the *pax6* in *Platynereis*. Concerning the r-opsin present in the *Platynereis* eyes, *pax6* is shown not to be the regulator of *r-ops1* in the *Platynereis* adult eyes.

To investigate the potential role of *pax2/5/8* transcription factor during the *Platynereis* eye development, CRISPR/Cas9 was successfully implemented and verified in *Platynereis* as a functional tool able to provide the *pax2/5/8* knockout.

KEYWORDS

eye development, transcription factors, *pax6*, *pax2/5/8*, CRISPR/Cas9, *Platynereis dumerilii*

ABSTRAKT

Genové regulační sítě zodpovědné za molekulární regulaci vývoje oka jsou evolučně konzervované napříč mnoha organismy. Zástupci genové rodiny transkripčních faktorů Pax patří mezi nejvíce konzervované zástupce těchto sítí. Pax transkripční faktory hrají klíčovou roli při vývoji zásadních součástí oka, přičemž jednou z jejich funkcí je i regulace opsinů - molekul zodpovědných za přeměnu světelného podmětu na elektrochemickou signalizaci ve fotoreceptorových buňkách. V rámci této diplomové práce jsou zkoumány geny *pax6* a *pax2/5/8* jako potenciální transkripční faktory regulující vývoj oka u modelového organismu *Platynereis dumerilii*.

Na základě sledovaného brzkého počátku exprese *pax6* a *pax2/5/8* byla testována možná role těchto transkripčních faktorů v regulaci r-opsinu. Expresní analýza *pax6* a *pax2/5/8* u divokého typu *Platynereis* je prováděna pomocí "whole mount" RNA *in-situ* hybridizace, společně s počáteční analýzou *Platynereis pax6* knockout linie. Ačkoliv má homozygotní mutace v *pax6* letální následky, heterozygotní mutanti přežívají a jsou schopní reprodukce. Uváděná data naznačují, že p*ax6* nereguluje u *Platynereis* geny *pax2/5/8, otx* a *six3.* Stejně tak *r-ops1,* tvořící r-opsin přítomný v dospělých očích *Platynereis*, není regulován pomocí *pax6*.

Pro výzkum potenciální role *pax2/5/8* transkripčního faktoru v průběhu vývoje oka *Platynereis* byl v rámci této práce zaveden CRISPR/Cas9 systém. Zavedení CRISPR/Cas9 systému pro tento modelový organismus bylo úspěšně ověřeno a systém byl prokázán jako schopný tvorby mutace *pax2/5/8* u *Platynereis*.

KLÍČOVÁ SLOVA

vývoj oka, transkripční faktory, *pax6*, *pax2/5/8*, CRISPR/Cas9, *Platynereis dumerili*

CONTENTS

LIST OF ABBREAVIATIONS

CRISPR – clustered regularly-interspaced short palindromic repeats

Cas9 – CRISPR associated protein-9 nuclease

DBD – DNA binding domain

EtOH - ethanol

ey – eyeless - *Drosophila Pax6* gene paralogue

eyg - eyegone - *Drosophila Pax6*-like gene

GRN – gene regulatory network

hpf – hours post fertilisation

MetOH - methanol

*otx/Otx/*Otx– orthodenticle homeobox

*pax/Pax/*Pax/PAX - paired box

*prox/*Prox – prospero homeobox

r-ops – r-opsin

six/Six - sine oculis homeobox

SNP – single nucleotide polymorphism

spa – sparkling - *Drosophila Pax2* homologue

TF – transcription factor

TriPaxB – Trichoplax paxB gene

toy –twin of *eyeless* - *Drosophila Pax6* gene paralogue

toe – twin of *eyegone* - *Drosophila Pax6*-like gene

WMISH – whole mount RNA in-situ hybridisation

wt – wild type

1. INTRODUCTION AND AIMS OF THE THESIS

Transcriptional regulation is considered to be highly conserved mechanism regulating gene expression across various organisms. This process is controlled through the operation of transcription factors, DNA binding proteins involved in the regulation of transcription of genes. Regulation of transcription enables unique expression of each gene in diverse cell types and during the embryonic development, which is crucial for the proper formation of organs. Development of the eye, as an organ providing vision, is regulated by many transcription factors, however, those from the Pax family are considered among the most conserved ones.

The thesis is focused on the data collection regarding the early eye development of non-classical model organism *Platynereis dumerilii*. Due to the *Platynereis* unique phylogenetic position within the Lopchotrochozoa lineage, subsequent utilisation of these data can give insight into the ancestral regulation of the eye development by comparison with Deuterostomia and Ecdysozoa.

To investigate the molecular regulation of the early eye development, aims of the thesis were set out as follows:

1. To provide the expression analysis of the *pax6* and *pax2/5/8* genes during the early development in the wild-type *Platynereis dumerilii*.

2. To provide the analysis of the *Platynereis pax6* knockout line regarding the gene dosage effect and analysis of *pax2/5/8, otx3, six3* and *r-ops1* genes during the *Platynereis* early development.

3. To implement the CRISPR/Cas9 system into the *Platynereis* to prepare the functional tool for the *Platynereis pax2/5/8* knockout line generation.

2. LITERATURE REVIEW

2.1 *Platynereis dumerilii* as a model organism in developmental biology

Platynereis is a relatively new emerging model organism important for the understanding of evolutionary relationships of organisms. Due to its unique position on the phylogenetic tree within the Lopchotrochozoa (Fig. 1), *Platynereis* is complementing a gap on the tree of life, since no other commonly used model organism is placed within this evolutionary lineage. Relatively easy breeding through the whole year with a large number of embryos and the possibility of genetic manipulations, make this animal becoming a very informative system for evolutionary developmental (evo-devo) biology studies. The eyes and nervous system are one of the most examined areas of the *Platynereis* research.

Various methods of molecular biology established in other model organisms, such as whole mount RNA in-situ hybridization (WMISH), morpholino knockdowns or t*ranscription activator-like effector nucleases (TALEN)* have been implemented in *Platynereis* recently. Nevertheless, new emerging methods, including the system of clustered regularly-interspaced short palindromic repeats (CRISPR) with associated protein-9 nuclease (Cas9), are still not introduced into this new model organism.

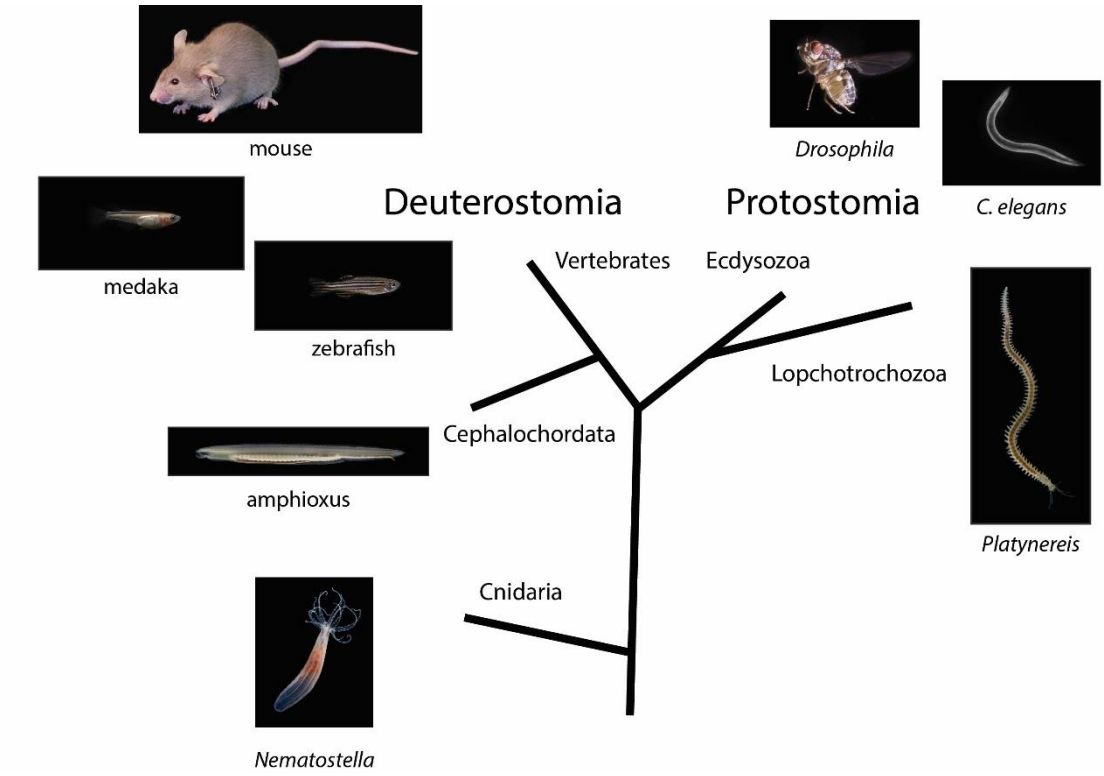

Fig. 1: Position of *Platynereis dumerilii* and selected commonly used model organisms on the tree of life. Common model organisms in developmental biology belong to vertebrates (mouse, zebrafish, medaka), Ecdysozoa (*Drosophila, Caenorhabditis elegans*), Cephalochordata (amphioxus) or Cnidaria (*Nematostella*) compared to *Platynereis*, which belongs to the Lopchotrochozoa lineage.
(picture of *C. elegans* adapted from www.usc.edu, picture of *Drosophila* adapted www.nationalgeographic.com)

2.1.1 Phylogenetic position of *Platynereis dumerilii*

Evolution of genetic machinery responsible for developmental processes is currently acknowledged to be a major field of the evolutionary developmental biology (evo-devo). Comparison of developmental processes at the molecular level provides insight into how the gene regulatory networks (GRNs) underling organismal development has evolved (Müller 2007). Analysis of different phylogenetic lineages and distantly related animals gives us broad perception of the evolution of genetic toolkits. However, according to the current classification of bilaterian animals to Deuterostomia, Ecdysozoa and Lophotrochozoa (Peterson and Eernisse 2001), well established model organisms for the molecular study of development are only located on two branches of this classification. Vertebrates (for example mouse, zebrafish or medaka) or Cephalochordata (amphioxus) belong to Deuterostomia and arthropods (*Drosophila*) and nematodes (*Caenorhabditis elegans*) to Ecdysozoa. The third branch - Lophotrochozoa is a notably low examined group in evolutionary developmental studies. Model organisms belonging to this group are not widely represented. Marine annelid *Platynereis dumerilii* is a progressing new model organism located within the Lophotrochozoa and is expanding a range of research possibilities in developmental comparative studies (Tessmar-Raible 2003).

As an added value, Lophotrochozoa are considered to be slow-evolving. In the question of their development, genes and body plans, they exhibit ancestral characteristics compared to other model organisms like *Drosophila* or *C. elegans* (Denes et al. 2007). Retention of ancestral gene features in *Platynereis* makes a suitable model for comparative developmental and evolutionary studies to find evolutionarily conserved characteristics in Bilateria. Comparison of *Platynereis* with vertebrates can also contribute to defining properties and characteristics of the last common ancestor of Protostomes and Deuterostomes (bilaterian animals) - the Urbilateria. Definition of properties and characteristics of urbilaterian is one of the major challenges of evo-devo (Tessmar-Raible 2003).

To sum up, preservation of ancestral characteristics and unique phylogenetic position within the Lopchotrozoa evolutionary lineage makes *Platynereis* a suitable model system for the evo-devo studies.

2.1.2 Life cycle of Platynereis dumerilii

Platynereis dumerilii is a widespread marine worm, reported from shallow coasts of North Africa to the Irish Sea, the Isefjord in Denmark and from the Mediterranean (Fischer and Dorresteijn 2004). About 35 mm full-size long animals are gonochoric (having separate sexes) and live mostly in self-spun tubes opened on both ends. These tubes are sized approximately as long as the worm´s body and are constructed on the ground surface from algae using the jaws or the anterior body segments (Daly 1973)

Life cycle (Fig. 2) takes up to 18 months, under laboratory conditions at least 3 months, but on average 6 or 7 months (Fischer et al. 2010). Developmental speed depends on the temperature of its environment, with *Platynereis* developing faster in higher temperatures. Linked to that, times mentioned in the text are given for the development at 18 °C (Fischer et al. 2010). Sexual maturation is controlled and synchronized by a lunar periodicity (Zantke et al. 2013), when atokous (immature) uniformly coloured juveniles transform into sexually dimorphic adults - yellow females and red-whitish males. Adult animals suspend their food income and undergo morphological change - their eyes increase in size and the trunk subdivides into two parts with different parapodia (Fischer et al. 2010). These sexually mature worms leave their tubes, rapidly swim in open water together with other mature worms and proceed to attract a sexual partner via pheromones (Zeeck et al. 1998). This behaviour results in the spawning ritual, when male and female swim in circle, release the sperm and eggs synchronously and both die after the spawning(Fischer et al. 2010).

One batch can consists of more than 2 000 eggs, which are transparent and measure about 160 μm in diameter. Eggs are enclosed by the protective jelly, self-produced during approximately first 40 minutes after a fertilisation. First cleavage appears after about 2 hours and is unequal, producing a pair of blastomeres about three-times larger than the pair of smaller ones. Second cleavage is meridional, equal in smaller blastomere and unequal in larger blastomere. Subsequent cleavages are more or less equatorial in a clockwise or counterclockwise manner, setting up a spiral cleavage motif. Blastomeres then rapidly divide resulting into a spherical mass, micromeres envelop the macromeres via the epibolic movements towards a vegetal pole and the number of cells quickly increases (Dorresteijn 1990; Fischer et al. 2010).

After approximately 13 hours, larvae begin to rotate slowly using cilliary band formed around their body. The ciliary band divides the animal to episphere - upper part; and hyposphere - lower part. Active swimming starts after 24 hours after fertilization, followed by the beginning of phototaxis at approximately 26 hours and accompanied with an appearance of a larval eye shading pigment. The pigment of adult eyes becomes clearly visible in about 51 hours. Another characteristic features used for the *Platynereis* stages classification and observation of the developmental progress are often connected with the nervous system and musculature. Lateral appendanges (parapodia) appear

at approximately 3 days. The beginning of the food intake starts around 5 days, when the animal´s gut becomes functional and worm uses up the embryonal lipid droplets and start feeding on algae from the environment. Until the start of the food intake, the development among larvae of one batch is highly synchronized (Fischer et al. 2010).

Larvae are freely swimming in the water until they reach breakthrough of three body segments, when they switch their lifestyle from pelago-benthing to fully benthic and settle on the ground, living in self-spun tubes.

Under laboratory breeding conditions, embryos could be obtained year-round. Relatively short generation time, easy breeding and a large number of transparent embryos are relevant benefits of *Platynereis* usage as a model organism.

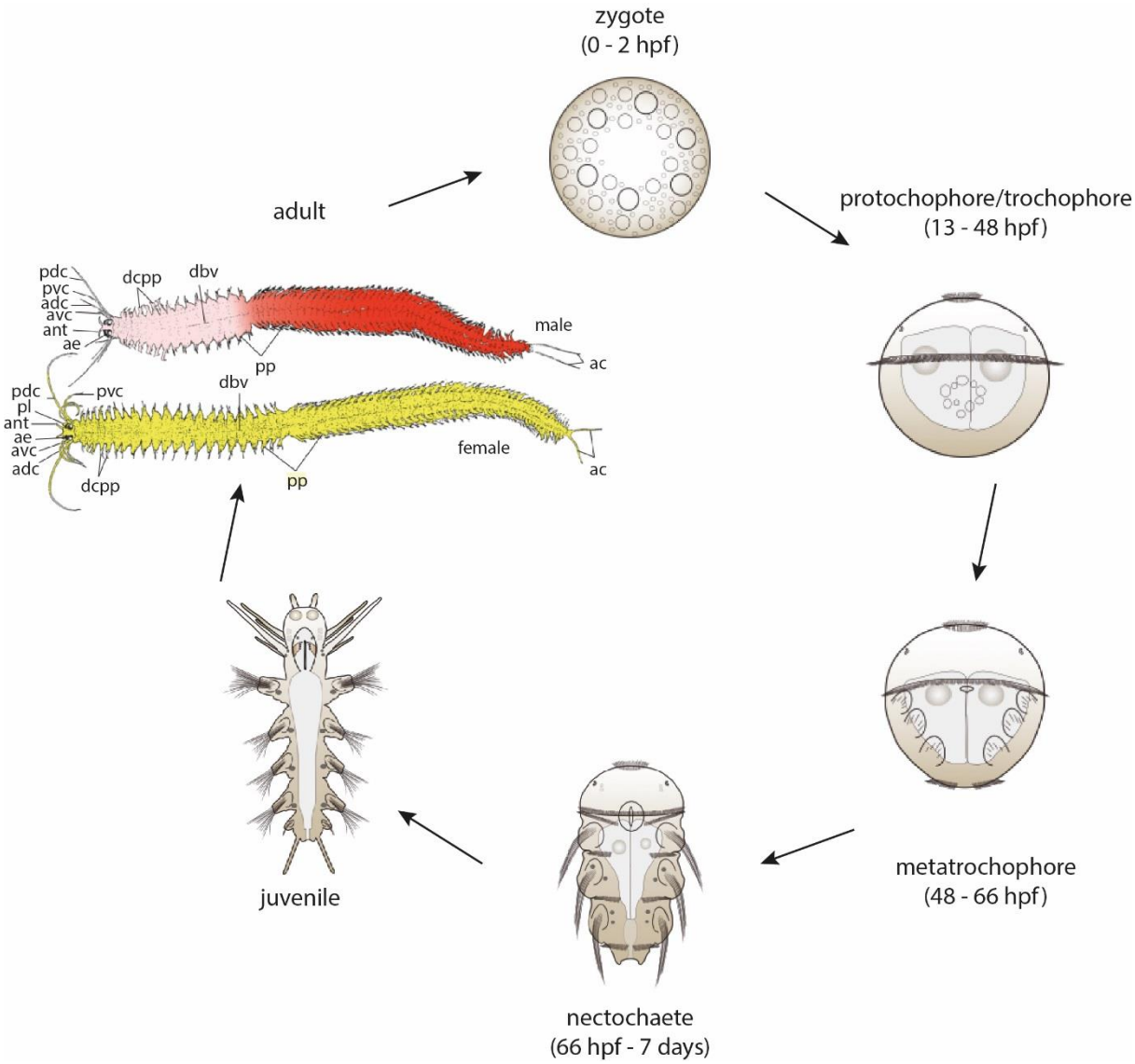

Fig. 2: Platynereis dumerilii life c*ycle.* (adapted from Fischer et al. 2010)
ac = anal cirrus, ant = antenna, adc = anterior dorsal cirrus, ae = adult eyes, avc = anterior ventral cirrus, dbv = dorsal blood vessel, , dcpp = dorsal cirrus of the parapodia pl = palpus, pp = parapodia, pvc = posterior venral cirrus

2.1.3 Larval morphology of investigated stages of *Platynereis dumerilii*

Stages of 24, 48 and 72 hours post fertilisation (hpf) were investigated during the thesis, which is linked to the start of the *Platynereis* eye development (Fig. 3). Majority of *Platynereis* staging systems refer to the animal´s age at hpf. However, *Platynereis* development is dependent on environment temperature and animals develop faster in higher temperatures. It is important to recognize main developmental features and landmarks to determine the proper developmental stage for comparison of individual animals. Currently used staging system is based on developmental landmarks together with time given at hours post fertilisation at 18 °C (Fischer et al. 2010).

Platynereis larvae start to swim actively in 24 hpf. Swimming is driven by the ciliary band made out of multiciliated cells of the prototroch. First larval nerve axon and first pigment in larval eyes appear, however larvae are not phototactic yet. Larval eyes are completely developed at 48 hpf, located laterally on the episphere. First chaetae are formed inside the trunk and three segments of the body appear simultaneously at 48 hpf also. Trunk rapidly elongates and a shape of larvae changes from spheroidal to conical and subsequently worm-like. Larvae parapodia move independently at 72 hpf and adult eyes are strongly pigmented. Larval eyes are still present at 72 hpf (Fischer et al. 2010).

Larval and adult eyes are easy recognizable developmental features. This makes them a helpful landmark in determination of developmental stages.

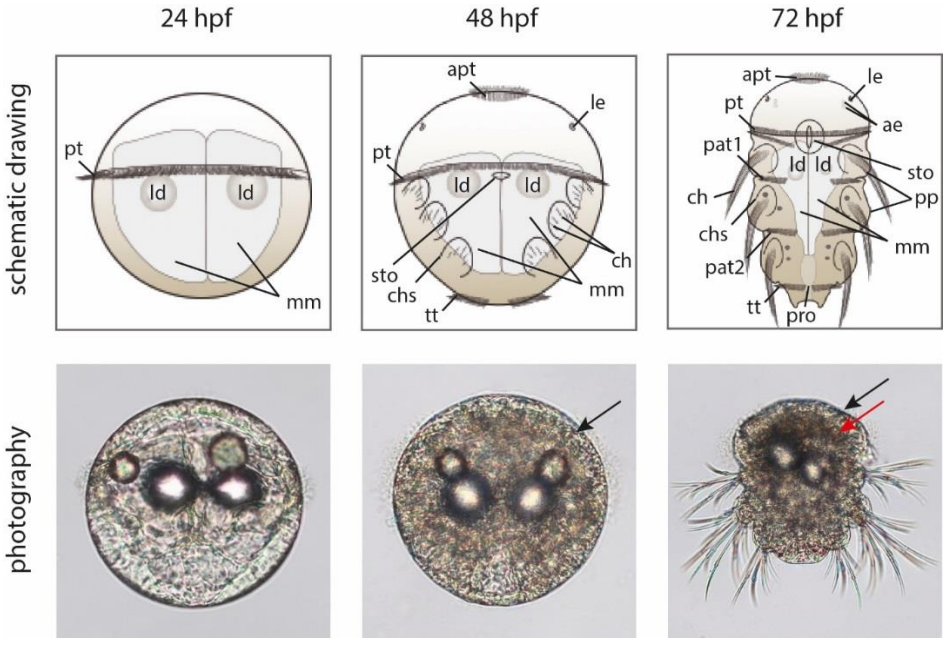

Fig. 3: *Platynereis* stages of 24, 48 and 72 hpf, schematic drawing and photographs. Larval eyes marked by black arrows, adult eyes marked by red arrows. Key morphological features labelled in schematic drawing by shortcuts (adapted from Fischer et al. 2010).
apt = apical tuft, ae = adult eyes ch = chaetae, chs = chaetal sac, le = larval eyes, ld = lipid droplets, mm = macromere, pp = parapodia, pro= proctodeum, pt = prototroch, pat1 = first paratroch, pat2 = second paratroch, sto = stomodeum, tt = telotroch

2.2 Eyes

There are many kinds of eyes present in the nature, varying in their anatomy, complexity, size or even function, such as image forming function compared to the processing of the light direction. The existence of an extreme variability in eye forms (from simple two-celled eyes up to the compound eyes of insects or camera-type eyes of vertebrates) may imply that eyes have evolved independently multiple times during the evolution. Contrary to this situation, distinct eyes share some basic features, most notably the same molecular toolkit, which may support the evolution from a common ancestor. Opsins, *Pax* – paired box or *Otx* – orhodenticle homeobox genes, can be viewed as an example of shared components (Arendt and Wittbrodt 2001; Gehring and Ikeo 1999). Together with shared features, situations when different genes were recruited to do similar tasks in different organisms are also present, such as in case of crystallins (lens proteins) or genes responsible for an eye shielding pigments (Vopalensky and Kozmik 2009).

All eyes require minimal framework, a photoreceptor containing photosensitive molecules and a shielding pigment. According to this requirement, simple eye can only be composed from two cells – one photoreceptor cell and one pigment cell (Fig. 4). Even the organ formed of single photosensitive cell containing photosensitive and shading pigment is considered to be an simple eye (Arendt and Wittbrodt 2001; Vopalensky and Kozmik 2009). Larval eyes of *Platynereis dumerilii* (Rhode 1992) or Hesse eye cups of amphioxus (Lacalli 2004) can be acknowledged as an example of two-celled simple eyes. This simple visual system was probably first employed for visual phototaxis (Randel and Jékely 2016).

Numerous ways of eye assembly, however, always based on photoreceptors in the vicinity of shielding pigment enable the organism to detect light, sense the light direction and, with connection to motor structure, also a movement in response to light.

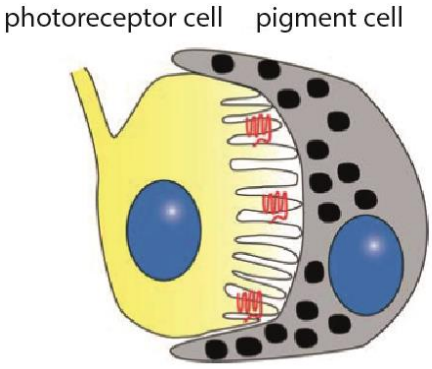

Fig. 4: Simple eye, schematic drawing (adapted from Vopalensky 2009).

2.2.1 Shielding pigment

Dark pigment provides additional information for sensing light direction by reduction of photon scatter, thus restricting the incoming light. Typically, pigment can be found in cells adjacent to photoreceptors. In simple eyes, the sensing of light direction is the main task of pigment granules. In more complex eyes accompanying functions are added - such as improvement of image quality due to the light absorption after its flow through the photoreceptors, together with protection of sensitive tissues. A huge set of dark-coloured molecules serves as shielding pigment in various types of eyes and often one type of eye contains more than one pigment type. As examples of eye pigments in different organisms can be listed melanin – in vertebrates (Bowness and Morton 1952), in planarians (Lambrus et al. 2015), or in *Drosophila* (Shoup 1966), ommochromes - in *Drosophila* (Ryall a Howells 1974; Shoup 1966), pterins – in *Drosophila* (Shoup 1966) or in *Platynereis dumerilii* (Viscontini M. et al. 1970). Shielding pigment is necessary component of minimal framework required for an eye function.

2.2.2 Photoreceptor

First step involved in a vision is a conversion of light (photon stream) into an electrochemical signal provided by the photoreceptor. According to the morphology of membrane surface, photoreceptor cells can be divided into two groups – rhabdomeric and cilliary. The group of rhabdomeric photoreceptors is forming microvilli on an apical cell membrane, whereas the group of ciliary photoreceptors is using folding of a membrane with cilia. Rhabdomeric photoreceptors are mostly present in the eyes of invertebrates, ciliary photoreceptors are principally used in the eyes of vertebrates (Fernald 2006). Microvilli or cilliary membrane folding provides enlargement of the membrane surface for the storage of an opsin photopigment involved in the light conversion (Arendt and Wittbrodt 2001).

Both types of photoreceptors use seven transmembrane receptors - opsins as light-sensitive proteins. As members of G-protein coupled receptors superfamily, opsins are coupled to Gα protein. Signal transduction processing and activation of an alpha subunit of a G-protein and following signal transmission is induced via conformational change of the opsin after it absorbs the photon. However, each photoreceptor class – rhabdomeric or ciliary, uses different phototransduction cascade (Fernald 2006). Besides visual purposes, opsins are present in a wide variety of tissue and cell types with great diversity. However, no major evolutionary group of opsins has the same genomic sequence linked to a specific tissue or cell type (Porter et al. 2012).

In the terms of the situation connected with visual systems, is that r-opsins (rhabdomeric opsins) are used primarily in rhabdomeric photoreceptors and c-opsins (ciliary opsins) are used

primarily in ciliary photoreceptors. However, expression of opsins from other opsin families, such as Go-opsins or retinochromes, which is also present in visual photoreceptors, makes the classification more complicated (Gühmann et al. 2015; Katagiri et al. 2001; Terakita et al. 1989). Derived rhabdomeric opsins can be found also in the vertebrate eye expressed in specific subtype of retinal ganglion cells called intrinsically photosensitive retinal ganglion cells (Graham et al. 2008). Ciliary opsins can be found in invertebrates as brain receptors (Arendt et al. 2004) .

Photoreceptors are an indispensable part of the eye required for the processing of information. Processing of information by opsin molecules in the photoreceptor cells is a crucial part of the visual sensory process.

2.3 Eyes of Platynereis dumerilii

2.3.1 Larval eyes

Platynereis possesses one pair of larval eyes located laterally on the episphere, first visible at 22 hours of development (Arendt et al. 2002). These eyespots are composed of the single rhabdomeric photoreceptor cell and a single pigment cell, lying within an epidermis close under the cuticle (Rhode 1992). This corresponds to the definition of a bilaterian two-celled prototype eye and is considered to be the simplest eye conserved across bilateralia (Gehring and Ikeo 1999).

Due to the orientation of this photoreceptor cell toward the concavity of a pigment cell, these larval eyespots are referred to as inverse eyes (Rhode 1992).

Distal part (apex) of a pigment cell contains dense granules of a shading pigment, which increases during larval development (Fig. 5) (Rhode 1992). During further development, eyes obtain their characteristic cup shape and a red pigment often appears. However, appearance and amount of pigment varies between batches and even between individuals (Fischer et al. 2010).

Reduction of larval eyes starts around the third day of development (Rhode 1992). Positive phototactic behaviour which started from 26 hours post fertilisation (at 18 °C) is further replaced by the vision provided by adult eyes (Fischer et al. 2010).

Larval eyes are an important visual organ during the first days of development of *Platynereis*, providing information about environmental light conditions (Fischer et al. 2010).

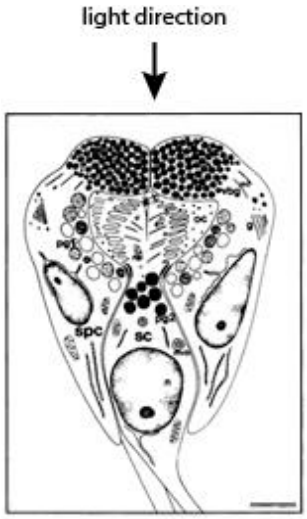

Fig. 5: *Platynereis* larval eye, schematic drawing, scale = 1 µl (adapted from Rhode 1992)
bb = basal body, cu = cuticle, ec = epidermal cell, g = *Golgi* cisternae, m = mitochondrion, mvb = multivesicular body, n = nucleus, pc = pigment cell, pg = pigment granule, sc = sensory cell, smc = submicrovillar cisternae

2.3.2 Adult eyes

Adult pigment-cup eyes represent a different type of eyes than the larval eyes. Adult eyes consist of two pairs of eyes latelar-dorsally in the larval episphere, one located more anteriorly compared to the other one (See Fig. 3 – 72 hpf). Development starts from the eye anlagen, each eye anlage gives rise to two adult eyes – one from the anterior and one from the posterior pair of eyes (Rhode 1992).

Around 40 hours after fertilisation the eye anlage consisting of four cells begins to develop directly under the cuticle (Fig. 6 and Fig. 7). Two peripherally situated supporting cells enclose two sensory cells. Later on, corneal cells differentiate and move between the cuticle and eye anlage, which goes down below the epidermis. The number of cells increases in each anlagen to four sensory and four supporting cells and during the following hours, the whole structure is reorganized to two pairs of supporting cells surrounding two pairs of sensory cells. Eye anlagen of the anterior eye is separated from the posterior one at 3 days old larvae, resulting into two separate pairs of eyes (Rhode 1992).

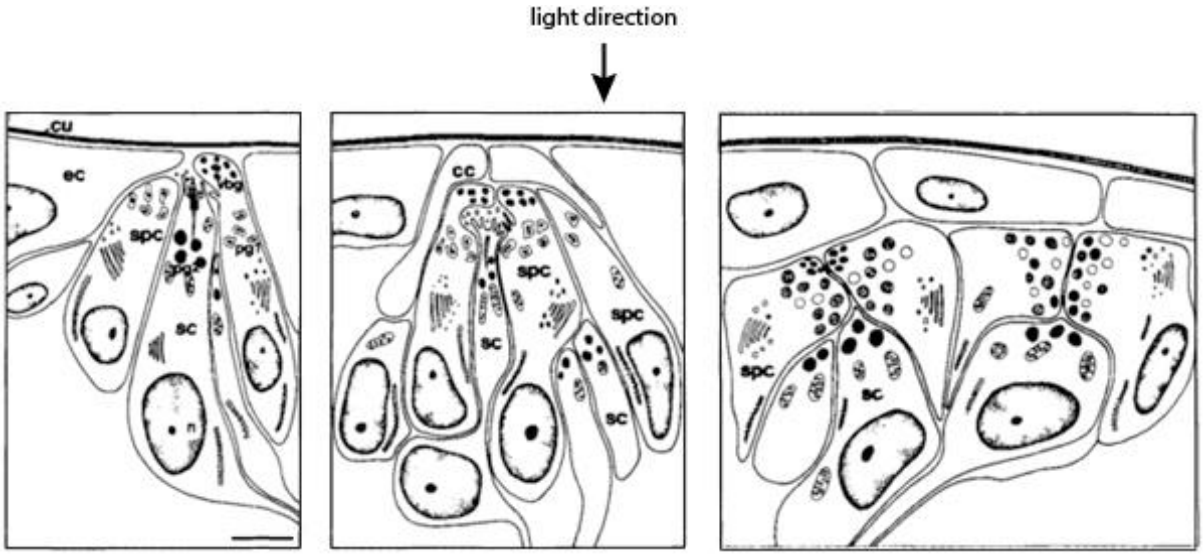

Fig. 6: *Platynereis* adult eye, development from 2 to 3-day old larvae, schematic drawing, scale = 2 µl (adapted from Rhode 1992)
cc = corneal cell, cu = cuticle, ec = epidermal cell, vbg = vitreous body granules, n = nucleus, pg 1, 2 = pigment granules type 1 and 2, sc = sensory cell, spc = supporting cell

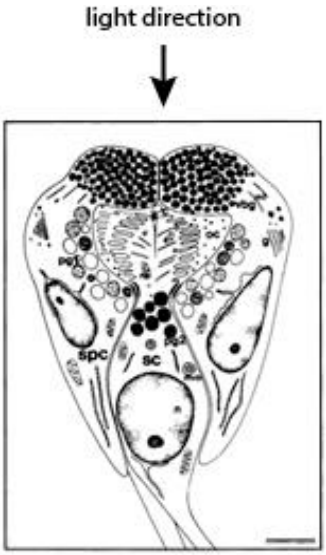

Fig. 7: Schematic drawing of one eye in 3 day old larva, longitudal section, scale bar = 2 µl (adapted from Rhode 1992)
g = *Golgi* cisternae, vbg = vitreous body granules, m = mitochondrion, mt = microtubules, mvb = multivesicular body, n = nucleus, oc = ocular cavity, pg 1, 2 = pigment granules type 1 and 2, sc = sensory cell, spc = supporting cell

The pigment cup of the eye is formed from the pigment granules of the supporting cells together with contribution of sensory cells pigment granules. Supporting cells perform transformation from spheroidal into tubular shape, enveloping the sensory cells and forming the ocular cavity under the pigment cup. New cells are added to the periphery of the pigment cup, contributing to the lens/vitreous body (Rhode 1992). Contrary to the larval eyes referred as inverse eyes, adult eyes are referred to as everse due to rhabdomeric photoreceptors oriented away from the concavity of pigment (Arendt et al. 2002).

The size of the adult eye is increasing during the development of *Platynereis*. The most apparent increase in the eye size occurs during the transformation of juveniles into sexually dimorphic adults (Rhode 1992).

Two pairs of adult eyes represent complex visual organs providing information income and perception. Although adult eyes are different type of eyes than the larval eyes, both play acritical role in the *Platynereis* visual sensory process.

2.4 Molecular regulation of eyes

Eyes were traditionally considered to be a clear example of a structures, which have evolved multiple times. Many different types of eyes from compound eyes of insects up to camera-type eyes of vertebrates seemed to be highly improbable to evolve from a common ancestor. Contrary to that, many genes playing role in the molecular regulation of the eye development were found to be highly evolutionarily conserved among many animal phyla. Research in *Drosophila* and mouse revealed homology of the transcription factor *Pax6*, which has a crucial function during the eye development in both animals. Moreover, *Pax6* amino acid sequence identity of about 90 % was found between *Drosophila* and mouse (Quiring et al. 1994, Hill et al. 1991) Other genes acting in the eye development gene regulatory networks (GRNs) are being investigated, revealing further insight into the evolutionary conservation of the eye molecular regulation.

Opsins, providing the conversion of the light into the electrochemical signalling in the cell as was previously mentioned, are essential molecules operating in the organism response to the light. There are two main types of opsins, r-opsin preferably used for the visual purposes in the eyes of invertebrates and c-opsin preferably present in the eyes of vertebrates (Fernald 2006). These opsins are considered to be derived from the ancestral opsin present in the Urbilateria - common ancestor of bilaterian organisms (Arendt a Wittbrodt 2001). Comparison the transcriptional regulation of the r-opsin and c-opsin enables us to determine shared components, which were supposedly present in the regulation of the urbilaterian opsin (ur-opsin).

Many transcription factors are considered to play role during the eye development. In the current hypothetical model transcription factors (TFs) from the Pax and Otx families seem to be shared components of the r-opsin and c-opsin regulation. Additionally to that, Prox – prospero homebox TF is considered to be co-opted for the regulation of r-opsin and Rx is regarded to be recruited for regulation of c-opsin (Fig. 8) (Vopalensky 2009). Regulation of development by TFs is a key aspect underlying the proper eye formation.

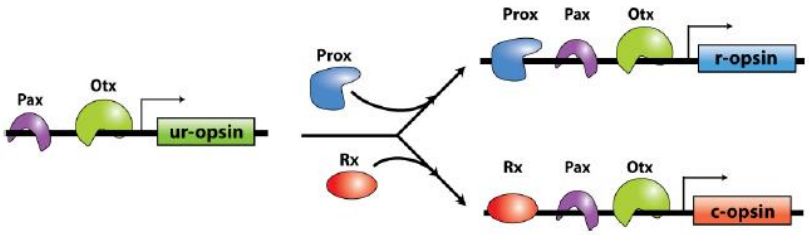

Fig. 8: Hypothetical model scheme of urbilaterian opsin regulation (adopted from Vopalensky 2009). TFs from the Pax and Otx families are presumed to be involved in the ur-opsin regulation, based on shared conserved binding sites within regulation sites of the r-opsin and c-opsin.

2.5 Transcription factors

Transcription factors (TFs) are proteins with the ability to bind to the specific genomic DNA sequences (Badis et al. 2009), acting as key regulators of gene transcription. Defining, which genes are going to be switched on and off, or more or less active at different time, TFs enable cells with the same genome to act differently. This plays an essential role in the regulation of development, cell differentiation and homeostasis. Moreover, elements which are recognized by TFs are conserved in different species of Eukaryots (Nitta et al. 2015).

TFs contain one or more DNA-binding domains (DBDs) allowing specific transcription modulation. There are various types of DBDs, however, they typically share diverse structural features and motifs, which are used for the classification of transcription factors according to the DBD sequence and structural similarities (Weirauch and Hughes 2011). DBD is critical for the TF function and mutation of one or a few crucial DBD aminoacids can change preferences for the binding to the specific DNA sequence (Aggarwal et al. 2010). Binding of DBDs to the DNA strand can be maintained via sequence specific interactions with DNA bases or depend on non-sequence specific interactions with the backbone of the DNA (Berg and von Hippel 1987; Jen-Jacobson 1997). TFs can act as transcription activators or repressors, but the effect depends on the area where the TF acts and also on other factors with which the TF operates, such as chromatin structure or interaction with specific proteins (Li et al. 2011; Yan et al. 2013). Cooperation among individual TFs is also a common feature in Eukaryots (Weirauch and Hughes 2011).

TFs exhibit DNA sequence binding preferences (Weirauch et al. 2014), however, binding can be influenced by competition or cooperation with other DNA-binding proteins (Wasson and Hartemink 2009). Other aspects affecting TF binding are the site accessibility within chromatin and interaction with chromatin modifications and additional chromatin proteins. According to that, TFs are not only considered to be affected by chromatin, but also on the other hand TFs are regarded as the chromatin environment establishing factors (Weirauch and Hughes 2011). Transcription factors binding performance can also be regulated by environmental conditions, such as stress (Harbison et al. 2004).

Within Eukaryota, TFs greatly vary in predominance in different organisms. However, greater number of TFs is present in larger genomes (Wilson et al. 2008). Moreover, proportion and total number of TF present in the genome is considered to be approximately corresponding to the organism complexity (Levine and Tijan 2003). The increase in the number of TFs probably enabled the origin of development of multicellularity connected with the ability to create new cell types (de Mendoza et al. 2013).

TFs are critical players in organismal development. Regulation of transcription by the TFs is essential for many processes within an organism and the regulation of TFs can be maintained by

various aspects. Nevertheless, specific transcription modulation is always provided by the binding to the DNA.

2.5.1 Pax transcription factors

Pax transcription factors (Fig. 9) are characterized by the presence of paired domain, a conserved 128 amino acid motif involved in the DNA binding (Bopp et al. 1986). This domain is made of two separate subdomains, N-terminal PAI domain and C-terminal RED domain providing bipartite binding to the DNA (Czerny et al. 1993). *Pax* genes produce different transcripts by alternative splicing according to spatiotemporal conditions. The resulting alternative isoforms differs in specifics of DNA binding and maintain various functions in further genetic regulation (Thompson and Ziman 2011).

Pax genes are probably derived from a common ancestral *Pax* gene. This is supported by the identification of one simple *Pax* gene – *TriPaxB* present in the morphologically simplest non-parasitical multicellular metazoan *Trichoplax adhaerens* (Hadrys et al. 2005). Subsequently, this ancestral *Pax* gave rise to the four *Pax* genes – *pax1/9, pax2/5/8, pax3/7* and *pax4/6,* which are found in basal chordates (Chen et al. 2010) and tunicates (Mazet et al. 2003). Based on that, Pax transcription factors are currently classified into four classes named after these genes, thus *Pax1/9, Pax2/5/8, Pax3/7* and *Pax4/6.* There are nine *Pax* genes (*Pax1-9*) present in mammals, resulting from further whole genome and later also partial duplications. The conservation of the functional paired domain can nevertheless be found across many animal phyla among diverse *Pax* orthologs (Weirauch and Hughes 2011).

Members of the Pax protein family play a critical role during organogenesis in many distinct organisms (Blake and Ziman 2014; Dahl et al. 1997), especially during the development of the brain, nervous system (Thompson and Ziman 2011) and eyes (Kozmik 2005).

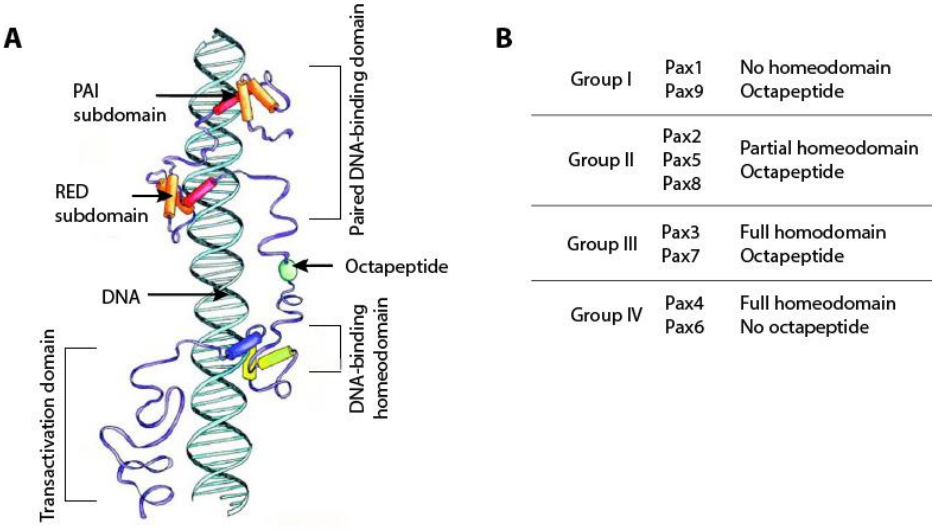

Fig. 9: A: All Pax proteins are characterized by the presence of paired domain, consisting of PAI and RED subdomains. Some of the Pax transcription factors has also an additional DNA-binding homeodomain and/or octapeptide region. B: Pax TF are classified to 4 groups - *Pax1/9, Pax2/5/8, Pax3/7* and *Pax4/6* (adapted from Blake a Ziman 2014).

2.5.1.1 Pax6 transcription factors

Pax6 was shown as the first factor responsible for eye development of both *Drosophila* (Quiring et al. 1994) and mouse (Hill et al. 1991). In both organisms, mutations in *Pax6* lead to the critical disruption of the eye development, resulting in the absence of eyes (Hill et al. 1991; Quiring et al. 1994).

There are two *Pax6* paralogs present in *Drosophila* – *eyeless* - *ey* (Halder et al. 1995) and twin of *eyeless* - *toy* (Czerny et al. 1999). *ey* and *toy* possess partially redundant function, both required for the development of structures derived from the *Drosophila* eye-antennal discs. From this eye-antennal discs are derived compound eyes and ocelli by the process of eye-antennal disc division into two distinct parts – eye field and antenna field. *ey* and *toy* expression can be initially found in the whole eye-antennal disc, however, the expression remains only in the eye field - giving rise to the compound eyes and ocelli, contrary to the antenna field, which is *ey* and *toy* negative (Quiring et al. 1994; Blanco et al. 2010; Kenyon et al. 2003).To establish the eye-antennal discs development, *toy* is involved in two pathways. The first pathway is direct *ey* activation by the *toy*, second pathway including the *optix* – a paralog gene of the *sine oculis,* is *ey* independent (Kronhamn et al. 2002). Knockout mutations of both, *ey* and *toy* are leading to the reduction of all structures derived from eye-antennal discs, resulting to headless flies (Kronhamn et al. 2002). Induced expression of *ey* leads to ectopic eyes development (Halder et al. 1995). Similarly induced expression of *toy* is leading to the ectopic eyes presence (Czerny et al. 1999). The normal expression of *toy* in mutants of *ey* gene suggests that *toy* runs upstream from *ey* in *Drosophila*. Moreover, the ectopic expression of *toy* resulted in the *ey* transcription at the ectopic expression site, contrary to the observation that misexpression of *ey* did not result in *toy* transcription in heterologous *Drosophila* imaginal discs (Czerny et al. 1999).

Except of *ey* and *toy* as homologues of *pax6* and *sine oculis*, two *Pax6*-like genes - *eyegone* - *eyg* (Jun et al. 1998) and *twin of eyegone* – *toe* (Yao et al. 2008) are considered to be the core genes of the *Drosophila* network maintaining the development of the eye primordium.

Similarly, *Pax6* is involved in the eye development of vertebrates. In zebrafish, *Pax6* have two paralogues genes – *pax6a* and *pax6b* (Nornes et al. 1998). *pax6b* is expressed in the retina and the early lens placode, accompanied by the expression in dorsal diencephalon, at the midbrain-hindbrain boundary and in the pancreas. *pax6a* expression in the lens and retina is followed by the expression in the developing diencephalon, telencephalon, hindbrain and spinal cord (Thisse and Thisse 2004). Simultaneous disruption of *pax6a* and *pax6b* function causes microopthalmia and general developmental delay of the zebrafish embryo (Kleinjan et al. 2008). Moreover, both genes were identified as having a significant role in the zebrafish retina regeneration (Thummel et al. 2010).

Mouse has single *Pax6* gene, which can be detected in all layers of the eye during the early development and subsequently in the developing neuroretina, retinal pigmented epithelium, cornea and the lens (Grindley et al. 1995; Walther and Gruss 1991). Gene dosage of *Pax6* is critical for the mouse eye development and homozygous mutants in *Pax6* have different phenotypes compared to heterozygotes. Homozygous *Pax6* knockout is embryonically lethal and the mice have several defects including absence of eyes and other defects in the head region (Grindley et al. 1995; Hogan et al. 1986, 1988). Mice carrying heterozygous *Pax6* mutations are viable but have several eye defects, including small eyes, lens developmental delay, cataracts (Collinson et al. 2001) or glaucoma (Kroeber et al. 2010).

PAX6 plays also a critical role during the eye development in human. Mutations in the PAX6 gene cause several malformations and diseases, such as anophtalmia (Glaser et al. 1994), aniridia (Ton et al. 1991), Peter´s anomaly (Hanson et al. 1994), congenital cataracts (Hanson et al. 1994) or autosomal dominant keratitis (Mirzayans et al. 1995).

Utilization of multiple *Pax6* gene transcripts resulting from alternative splicing variants allows Pax6 proteins to carry out different functions during the development. In mammals, there are three diverse isoforms of the Pax6 protein with different binding specificity, resulting in products of transcription driven by diverse *Pax6* promoters. Apart from the Pax6 classic/normal isoform, there is Pax6ΔPD isoform lacking the N-terminal paired domain and Pax6(a5), which binds to DNA using the RED and homeodomain without the functional PAI domain.

Pax6 products comparable to the vertebrate Pax6 protein and Pax6(5a) produced by alternative splicing can be found also in *Drosophila*. However, contrary to vertebrates, *Drosophila* Pax6(5a) is not a product of the alternative splicing, but a product encoded by a separate gene mentioned above - *eyg* and *toe*. Interestingly, two *pax6* gene loci – *pax6.1* and *pax6.2* were identified also in the elephant shark. In this case, *pax6.1* locus is highly similar to the mammalian *Pax6,* while *pax6.2* encodes the protein without the paired domain – similar to the mammalian Pax6 isoform Pax6ΔPD (Ravi et al. 2013).

Pax6 is essential transcription factor involved in the eye development and mutations in the *Pax6* gene lead to the eye defects. Amount and degree of these defects were found to be often dependent on the Pax6 dosage. Role of the *Pax6* is highly conserved across many different organisms, from *Drosophila* to human.

2.5.1.2 Pax2/5/8 transcription factors

Pax2 gene is considered to be another *Pax* gene possessing exclusive function during the eye development. *Pax2* has evolved from the *Pax2/5/8* gene which is present in basal chordates (Chen et al. 2010), tunicates (Mazet et al. 2003) or in molluscs (Wollesen et al. 2015). *Pax2/5/8* expression was found in photoreceptive organs of moluscs, confirming the role of pax2 within the lopchotrochozoan light-sensing systems (Wollesen et al. 2015).

Pax2 homologue in *Drosophila*, named *sparkling – spa,* is expressed in cone cells, primary pigment and bristle cells in eye discs of developing larvae and pupae. Development and assembly of cone and primary pigment cells is affected in the *Drosophila spa* mutants, leading to the altered number, shape, interactions and to the dissorganisation of ommatidial cone and pigment cells (Fu and Noll 1997).

In zebrafish, there are two *pax2* genes present due to the genome duplication in the teleost fish lineage, *pax2a* and *pax2b* (Pfeffer et al. 1998). Non-sense mutation in the zebrafish *pax2a* leads to the defect in the formation of the optic stalk resulting in the open optic fissure, accompanied by the loss of midbrain-hindbrain boundary, malformations in the nephric system and other defects. Expression of *pax2b* is independent of the *pax2a*, however, Pax2b protein is not able to compensate for the loss of *pax2a.* (Brand et al. 1996)

Pax2 also has an important role during the eye development in mouse. Its expression can be initially found in the ventral part of the optic evagination and subsequently after the formation of the optic cup. The expression extends dorsally into the optic stalk, marking the future optic nerve. *Pax2* is critical for the morphogenesis of the mouse eye, such as for close of the optic fissure and also for its regional specification, such as for the determination of the proximodistal axis of the optic evagination. *Pax2* mouse mutants have significantly abolished development of the optic nerve which exhibits the pathfinding defects. Together with the optic nerve defects, glial cells derived from the optic stalk are lacking and mutants do not form optic chiasma. *Pax2* mutant embryos show expansion of the *Pax6*-expressing retinal pigmented epithelium up to the optic cup/optic stalk boundary (Torres et al. 1996).

Mutations in PAX2 in humans are associated with the coloboma of the optic nerve (Sanyanusin et al. 1995) or renal coloboma syndrome, which is accompanied by several eye malformations, such as an optic nerve dysplasia, retinal coloboma or microphtalmia (Schimmenti 2011).

The expression of *Pax2* is often mutually exclusive to the expression of *Pax6* in optic systems. Undifferentiated *Drosophila* eye disc epithelium retains morphogenetic furrow, wherein *ey* is expressed anteriorly and *spa* is expressed posteriorly. (Fu a Noll 1997). Similarly, during the mouse development, *Pax2* is expressed in the tips of the optic fissure and in the optic stalk, forming a boundary to the expression of the *Pax6* in the pigmented retina of the optic cup (Torres et al. 1996).

Pax2 expression domains are expanding in the *Pax6* loss-of-function mutants and vice versa. This reciprocal transcriptional repression during the development of the mouse eye, interaction of the *Pax2* and *Pax6* establishes the regionalization and subsequent boundary foundation between the optic cup and optic stalk (Schwarz et al. 2000).

Mutations in *Pax2/5/8* and in its *Pax2* homologues cause defects in eye and optic nerve development in diverse organisms. *Pax2* was found to be reciprocally repressed on transcriptional level by *Pax6* in mouse, however, no other interaction was analysed in other animals. Pax5 and Pax8 homologues of Pax2/5/8 were not detected to play significant role during the eye development.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 List of bacterial strains

Bacterial strain *Escherichia coli* TOP10 *Invitrogen*

3.1.2 List of cultivation media

Bacterial LB medium– LB Broth *Sigma-Aldrich*

3.1.3 List of antibiotics

ampicilin 100 μg/ml *Sigma-Aldrich*

kanamycin 100 μg/ml *Sigma-Aldrich*

3.1.4 List of enzymes

proteinase K recombinant PCR grade *Roche*

restriction enzymes BamHI 20 U/ml, BsmBI 10 U/ml, EcoRI 20 U/ml, EcoRV 20 U/ml, HinDIII 20 U/ml *New England Biolabs*

SP6 RNA polymerase, T7 RNA polymerase *Roche*

T7 endonuclease *New England Biolabs*

3.1.5 List of commercial kits

6x DNA loading Dye T*hermo Scientific*

AccuPrime Pfx SuperMix *Invitrogen*

DreamTaq Green PCR Master Mix (2X) *Thermo Scientific*

DIG RNA Labelling Mix *Roche*

JetStar®2.0 Plasmid Purification Kit *Genomed*

NBT/BCIP substrate *Roche*

Marker GeneRuler DNA ladder Mix *Thermo Scientific*

QIAprep®Spin Miniprep Kit 250 *Qiagen*

QIAEX® Gel extraction Kit *Qiagen*

QIAquick® Gel Extraction Kit *Qiagen*

Quant-iT™ RNA Assay Kit *Invitrogen*

MegaShortScript™Kit *Invitrogen*

SuperScript® VILO™ cDNA Synthesis Kit *Invitrogen*

Vector® blue substrate Kit *Vector laboratories*

Zero Blunt®TOPO® PCR cloning Kit *Invitrogen*

3.1.6 List of plasmids

pCR®-BluntII-TOPO® *Invitrogen*

pT7-gRNA, addgene #46759, obtained from Z. Kozmik

3.1.7 List of primers

oligonucleotide name	**sequence 5´→ 3´**	**oligonucleotide description**
ID1	TCACGGTGGAGTCAACCAAT	*pax2/5/8* 1st exon genotyping primer forward
ID2	ACACAGCCATGGGAAACTCT	*pax2/5/8* 1st exon genotyping primer reverse
ID5	TAGGGAAGACCTCTCCCAGACG	*pax2/5/8* 1st exon CRISPR oligonucleotide forward
ID6	AAACCGTCTGGGAGAGGTCTTC	*pax2/5/8* 1st exon CRISPR oligonucleotide complement forward
ID7	TAGGCGACCATGCGACATCTCA	*pax2/5/8* 1st exon CRISPR oligonucleotide reverse
ID8	AAACTGAGATGTCGCATGGTCG	*pax2/5/8* 1st exon CRISPR oligonucleotide complement reverse
ID27	CTACGAGACAGGCTCAGTCC	*pax2/5/8* 2nd exon genotyping primer forward
ID28	GAACTAACGCTTGGGACATTTT	*pax2/5/8* 2nd exon genotyping primer reverse
ID30A	TAGGTTGGTGGCTCCAAGCCCA	*pax2/5/8* 2nd exon CRISPR oligonucleotide forward
ID30B	AAACTGGGCTTGGAGCCACCAA	*pax2/5/8* 2nd exon CRISPR oligonucleotide complement forward
ID31A	TAGGGGACCGGCTACTGGCTGA	*pax2/5/8* 2nd exon CRISPR oligonucleotide reverse
ID31B	AAACTCAGCCAGTAGCCGGTCC	*pax2/5/8* 2nd exon CRISPR oligonucleotide complement reverse
ID36	GCCAACCAGATGGTCAA	*α-tubulin* genotyping primer forward
ID37	GCTTGGTCTTGATGGTG	*α-tubulin* genotyping primer reverse
ID42ZK1257A	TTTCAGGTGTCGAACGGTTGT	*pax6* genotyping primer forward
ID43ZK1257F	ACAACAGCCTGTCCCCATATC	*pax6* genotyping primer reverse
ID47	ACGCTGGGTTTTACCAGATG	*r-ops1* gene expression onset primer forward
ID48	CCTTTTTGTGGAATCGAGGA	*r-ops1* gene expression onset primer reverse
ID49	CAGCTCAGCCATCATCAAGA	*prox1* gene expression onset primer forward
ID50	GGCGGGAGTATTTCTCCATT	*prox1* gene expression onset primer reverse
ID51	CTCCAGTTTCCCCAACTCAA	*pax6* gene expression onset primer forward
ID52	GTACAACGTGCCGATTGTTG	*pax6* gene expression onset primer reverse
ID53	GGTCGGAAACTGGTTCAAGA	*six3* gene expression onset primer forward
ID54	GGTGGCTAATGTTCGATGCT	*six3* gene expression onset primer reverse
ID55	CTTCAGGGCAGTACCACCAC	*otx* gene expression onset primer forward
ID56	ACCATATTGAGGGCGAGTTG	*otx* gene expression onset primer reverse
ID57	CCACACCACTTGGGACTTCT	*pax3/7* gene expression onset primer forward
ID58	TTTTGGAGCATGGTGGTACA	*pax3/7* gene expression onset primer reverse
M13 reverse	CAGGAAACAGCTATGAC	sequencing primer

3.1.8 List of antibodies

anti-DIG/AP *Roche*, dilution 1:4000

anti-acetylated α-tubulin antibody T6793 *Sigma-Aldrich*, dilution1:500.

Alexa Fluor® anti-acetylated tubulin secondary antibody *Life Technologies*, dilution 1:500

3.1.9 List of chemicals

TRIzol® *Life Technologies*

chloroform *Penta*

isopropanol *Penta*

agarose *Serva*

ethanol - EtOH *Penta*

ethidium bromide - EtBr *Sigma-Aldrich*

methanol - MetOH *Penta*

restriction enzymes buffers 2.1, 3.1 *New England Biolabs*

triethanolamine – TEA *Sigma-Aldrich*

10x PCR buffer DyNAzyme *Finnzymes*

3.1.10 Solutions composition

10xPBS

18.6 mM $NaH_2PO_4 \cdot H_2O$ (2,56 g/l)
84.1 mM $Na_2HPO_4 \cdot 2H_2O$ (14,97 g/l)
1.750 mM NaCl (102,2 g/l)
Phosphates mixed in 800 ml dH2O, pH adjusted to 7.4 (within 0.4) with HCl or NaOH.
Solution was DEPC treated and autoclaved. Storage at 4 °C.

PTw
100 ml 10x PBS stock
895 ml dH2O
5 ml 20% Tween-20
Solution was DEPC treated and autoclaved. Tween subsequently added.
Storage at room temperature.

4 % PFA
10 ml 16% PFA
30 ml PTw
Solution was filtered using 0.45 µm filter. Storage at -20 °C.

Miniprep solutions
P1: 1% glukose, 25mM TRIS pH 8.0, 10mM EDTA
Autoclaved. Storage at room temperature.
P2: 0,2M NaOH, 1% SDS

Storage at room temperature.
P3: 3M potassium acetate, 11.5% acetic acid
Autoclaved. Storage at room temperature.
TE: 10 mM TRIS pH 8.0, 1 mM EDTA pH 8.0
TER: TE: 10 mM TRIS pH 8.0, 1 mM EDTA pH 8.0, RNase 0.1 mg per 1 ml of solution

1x TAE buffer
to 1l of dH2O
48.4 g TRIS
20 ml 0.5 M EDTA pH 8.0
11.4 ml acetic acid
Storage at room temperature.

Hybridization buffer
20 ml formamide
10 ml 20x SSC pH 4.5, DEPC treated and autoclaved
0.1 ml heparin (20 mg/ml), made out of DEPC treated water
0.5 ml 20% Tween20
2 ml 20 % SDS
0.2 ml salmon sperm DNA
7.5 ml dH_2O
Storage at -20 °C.

20x SSC
175.3 g NaCl
88.2 NaCitrate (tribasic dihydrate)
1l dH_2O
Solution was DEPC treated and autoclaved. Tween (0.1 %) subsequently added to the WMISH solutions.
Storage at room temperature.

TRIS buffer
60.57 g TRIS
500 dH2O

AP+ buffer solution
2.5 ml TRIS pH9.5 (1M)
5 ml NaCl (1M)
2.5 ml $MgCl_2$ (1M)
2.5 ml 20% Tween
dH_2O up to 50 ml

AP- buffer solution
2.5 ml TRIS pH9.5 (1M)
5 ml NaCl (1M)
2.5 ml 20% Tween
dH_2O up to 50 ml

3.1.11 Other material

bacterial plates of 10 cm diameter *Gamedia*

borosilicate glass capillaries *Harvard Apparatus*

inoculation loops *Sarstedt*

injection syrings *Henke-Sass Wolf GmbH*

microscopy glasses *Knittel Gläser*

plastic pestle homogenizators Microtube Pellet Pestle® Rods *Kontes*

4 well plates for tissue cultures *TPP*

24 well plates for tissue cultures *TPP*

plastic tubes 1.5 ml *Eppendorf*

plastic tubes for PCR 0.2 ml *Axygen*

plastic tubes for PCR 0.5 ml *Axygen*

plastic filters Minisart HighF low 0.45 µm *Sarstedt*

transfer pipettes *Sarstedt*

pipette tips *Axygen*

3.1.12 List of used lab equipment

horizontal electrophoresis set-up *BIO-RAD*

horizontal electrophoresis electrical source *BIO-RAD* PowerPac^{TM}300

fluorometer *Quibit^{TM} Invitrogen*

camera *Canon EOS 30D*

camera *Canon EOS 1100D*

camera *Olympus DP72*

camera lens Canon EF 50mm f/1.8

centrifuge *Eppendorf MiniSpin plus®*

cooling centrifuge *Eppendorf Microcentrifuge 5424 R*

cooling centrifuge LWD 0.52 *Hettich Zentrifugen*

inverted fluorescent microscope *Leica DM1600* with confocal adapter *Leica TCS SP5 AOBS Tandem*

stereozoom microscope SZX9 *Olympus*

fluorescence lamp URFL-T *Olympus*

stereozoom microscope SZX7 *Olympus*

inverted microscope with fluorescence cubes *Diaphot 300, Nikon*

inverted microscope *Eclipse TS100 with ELWD 0.3 objective selector, Nikon*

set of pipets *Discovery Comfort BioTech*

water bath *Grant TECRA*

vortex *MS 1 Minishaker IKA*

printer of agarose gels photos *Mitschubishi 95*

thermostat TCH 100 *Laboratorni pristroje Praha*

thermal cycler *T100^{TM} BIO-RAD*

thermal block *THERMO BLOCK TDB-120 Biosan*

thermo shaker *Thermo-Shaker PSC24N PHMT Grant Bio*

spectrophotometer *NanoDrop ND1000*

rocking table *Kavalier LT2*

UV-transilluminator *UviTec Cambridge, BioTech*

gel documentation system *UviTec UVIdoc HD2 BioTech*

miliQ water generator *miliQ IWA20iol*

cooling incubator Q-Cell, *PolLab*

pressure injector FemtoJet *Eppendorf*

micromanipulator TransferMan NK *Eppendorf*

3.2 Methods

3.2.1 Obtaining animals

Adult and larvae *Platynereis dumerilii* animals were obtained from laboratory breeding cultures of Department of Transcriptional Regulation (Institute of Molecular Genetics of the ASCR, v. v. i., Prague) and Gáspár Jékely Lab (Max Planck Institute for Developmental Biology, Tuebingen).

3.2.2 Determination of the gene expression onsets of selected eye-specific genes in *Platynereis*

RT-PCR was performed to determine the expression onsets of selected eye-specific genes in Platynereis. RNA was isolated from different developmental stages of Platynereis and subsequently transcribed to cDNA using the reverse transcription reaction. Result DNA was used as a template for the RT-PCR experiment

3.2.2.1 RNA isolation

Material: TRIzol® (*Life Technologies*), chloroform (*Penta*), isopropanol (*Penta*), Quant-iT^{TM} RNA Assay Kit (*Invitrogen*), different developmental stages of *Platynereis* for each sample, agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*)

Solutions: 75 % EtOH, 1x TAE buffer

Equipment: cooling centrifuge *Eppendorf Microcentrifuge 5424 R,* vortex *MS 1 Minishaker IKA,* thermo shaker *Thermo-Shaker PSC24N PHMT Grant Bio,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac^{TM}300, UV-transilluminator *UviTec Cambridge, BioTech,* fluorometer *Quibit^{TM} Invitrogen*

Method:

All steps were performed under RNAse free conditions. 1 ml of Trizol reagent was added per 0.1 g of embryos and embryos were homogenized using a plastic pestle. For better homogenization, the process was split into two parts: firstly 50 µl of TRIzol was added, then the first homogenization was performed and secondly, with the addition of the rest of TRIzol volume, the specimen was homogenized once again. After waiting 5 minutes, 200 µl of chloroform was added (per 1 ml of TRIzol) and the mix was vortexed for 15 seconds. Tube was centrifuged 12 000 g at 4 °C for 15 minutes. Water phase on the top of the tube was transferred into new tube with 500 µl of isopropanol. After 10 minutes of waiting and following 10 minutes of centrifugation 12 000 g at 4 °C, the supernatant was removed and RNA pellet was wash with 1 ml of 75% EtOH. EtOH was removed after short spin and RNA pellet was left dry at room temperature. Completely dry pellet was dissolved in miliQH_2O and shaken for 10 minutes.

Quality of isolated RNAs was checked using horizontal gel agarose electrophoresis using 2% agarose gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 15 minutes at 130 V. Products were visualized using the UV-transilluminator.

Sample concentrations were measured by Quibit fluorometer, using Quant-iT™ RNA Assay Kit. Samples were stored at -80 °C in aliquots.

3.2.2.2 cDNA preparation

Material: isolated RNA samples from different developmental stages of *Platynereis*, SuperScript® VILO™ cDNA Synthesis Kit (*Invitrogen*)

Equipment: thermal cycler *T100™ BIO-RAD*

Method:

500 ng of RNA from each stage was used in the cDNA preparation. Reverse transcription reactions were prepared according to the following table and the following program was used for the cDNA synthesis.

Reaction mix:

VILO reaction mix	4 µl
enzyme	2 µl
RNA	500 ng
DEPC H_2O	12.5 µl

cDNA synthesis program:

incubation 25 °C	10 minutes
incubation 42 °C	60 minutes
termination 85 °C	5 minutes
final cooling 4 °C	"forever"

Samples were stored at -20 °C in aliquots.

3.2.2.3 RT- PCR

Material: cDNA synthetized out of the RNA isolated from different developmental stages of *Platynereis*, SuperScript® VILO™ cDNA Synthesis Kit (*Invitrogen*), agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*)

Solutions: 1x TAE buffer

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, UV-transilluminator *UviTec Cambridge, BioTech*

Method:

RT-PCR reaction was prepared according to the following table and run according to the following program. As a DNA templated was used cDNA synthetized out of the RNA isolated from different developmental stages of *Platynereis,* primers were designed using the Primer3 software.

Reaction mix:

DreamTag Green MasterMix	12.5 µl
miliQH_2O	10.5 µl
primers	1 µl
cDNA	1 µl

RT-PCR program:

95 °C	1 min
95 °C	*30 s*
60 °C	*30 s*
72 °C	*1 min*
	35x
72 °C	5 min
12 °C	„forever“

Result products after RT-PCR reaction were visualized and analysed using horizontal gel agarose electrophoresis using 2% agarose gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 45 minutes at 110 V. Products were visualized using the UV-transilluminator.

3.2.3 Gene expression analysis of *pax2/5/8, pax6* and analysis of *Platynereis pax6* knockout line

To provide gene expression analysis of wild-type *pax2/5/8* and *pax6* in *Platynereis*, accompanied with the initial analysis of *Platynereis pax6* knockout line, whole mount *in-situ* hybridization (WMISH) was employed. For the *pax2/5/8*, *otx* and *six3* were used own designed WMISH probes, prepared using the molecular cloning in *Escherichia coli* followed by the *in-vitro* transcription.

3.2.3.1 Molecular cloning in *E. coli*

3.2.3.1.1 Ligation and *E.coli* TOP10 transformation

Material: competent cells - bacterial strain *Escherichia coli* TOP10 (*Invitrogen),* bacterial LB medium– LB Broth (*Sigma-Aldrich),* bacterial plates of 10 cm diameter with agar (*Gamedia),* bacterial loops (*Sarsted),* antibiotic (ampicilin/kanamycin, 100 µg/ml), Zero Blunt®TOPO® PCR cloning Kit (*Invitrogen*)

Equipment: thermostat TCH 100 (*Laboratorni pristroje Praha),* water bath 42 °C

Method:

Ligation mixture was prepared according to the following table.

Ligation reaction mix:

salt solution	0.5 µl
plasmid pCR®-BluntII-TOPO®	0.5 µl
insert	2 µl

Ligation mix was to used to the *E.coli* TOP10 competent cells transformation. *E.coli* TOP10 competent cells were defrosted on ice and to the mixture of 100 ul of cells was added 1 µl of the ligation mixture. Competent cells were subsequently exposed to the heat shock at 42 °C for 30 seconds and then put back on ice immediately. In case of plasmids with kanamycin resistence were added 200 µl of LB medium without antibiotic after a few seconds in ice, cells were gently shaken for 40 minutes at 37 °C, subsequently inoculated onto the agar plates with kanamycin and incubated overnight at 37 °C. In case of plasmids with ampicillin resistance, cells were inoculated onto the agar plates with ampicillin directly after a few seconds in ice and incubated overnight at 37 °C.

3.2.3.1.2 Minipreparation of plasmid DNA (Miniprep)

Material: competent cells - bacterial strain *Escherichia coli* TOP10 (*Invitrogen),* bacterial LB medium– LB Broth (*Sigma-Aldrich),* isopropanol *(Penta*), antibiotic (ampicilin/kanamycin, 100 µg/ml)

Solutions:

P1 solution: 25 mM TRIS, 10 mM EDTA, 1% glucose, autoclaved

P2 solution: 0.2 M NaOH, 1% SDS

P3 solution: 3 M potassium acetate, 11.5% acetic acid

TER solution: 10 mM TRIS, 1 mM EDTA, RNase enzyme

80% EtOH

Equipment: vortex *MS 1 Minishaker IKA,* thermo shaker *Thermo-Shaker PSC24N PHMT Grant Bio,* centrifuge *Eppendorf MiniSpin plus®*, spectrophotometer *NanoDrop ND1000*

Protocol:

Unless stated otherwise, all steps were performed at room temperature.

1.5 ml of LB medium with 1.5 µl of antibiotic (dilution of antibiotic 1:1000) was inoculated by the single bacterial colony previously grown on agar plate and cultivated in plastic tubes on shaker at 37 °C overnight. The solutions were transferred next day into eppendorf tubes and centrifuged 10 500 g for 1 minute. After the supernatant discard, the pellet was resuspended in 300 µl of P1 solution and then mixed by the vortex machine. Then, 300 µl of P2 solution were added and the tube content was carefully mixed by the tube inversion. After 3 minutes were added also 300 µl of P3 solution and the mix was mixed by the vortex again. After 10 minutes of waiting the content was centrifuged 20 800 g for 10 minutes. 1 ml of supernatant from each tube was carefully transferred into clean tube with 500 µl of isopropanol and then mixed by the tube inverting. After 5 minutes, content was centrifuged 18 000 g for 10 minutes. Resulting pellet was washed by 250 µl of 80% EtOH and left drying. Completely dry pellet was dissolved in 30 – 50 µl of TER solution according to its size, and shaken 10 minutes at 37 °C.

Concentration of isolated plasmid DNA was measured by the spectrophotometer *NanoDrop ND1000*

Samples were stored at -20 °C.

3.2.3.1.3 Midipreparation of plasmid DNA (Midiprep)

Material: competent cells - bacterial strain Escherichia coli TOP10 (*Invitrogen),* bacterial LB medium– LB Broth (*Sigma-Aldrich),* JetStar®2.0 Plasmid Purification Kit (*Genomed*), isopropanol (*Penta*), antibiotic (ampicilin/kanamycin, 100 µg/ml), miliQH_2O

Solutions:

E1 - E6 solutions

70% EtOH

Equipment: cooling centrifuge LWD 0.52 *Hettich Zentrifugen,* spectrophotometer *NanoDrop ND1000*

Method:

Unless stated otherwise, all steps were performed at room temperature.

50 ml of LB medium with 50 µl of antibiotic (dilution of antibiotic 1:1000) was inoculated by the single bacterial colony previously grown on agar plate and cultivated in plastic tubes on shaker at 37 °C overnight.

Overnight LB culture was centrifuged 5 000 g for 10 minutes and the pellet was dissolved at 4 ml of E1 cell resuspending buffer. 4 ml of E2 cell lysis buffer was added after the dissolution of the pellet, content was carefully mixed by the tube inverting and then incubated for 5 minutes. 4 ml of E3 precipitation buffer was added, mix was shaken several times until it was homogenous

and then centrifuged 5 000 g for 10 minutes. Supernatant was then loaded onto previously E4 equilibrated column, left pass through and the column was washed twice by the E5 washing buffer. After the washing, the DNA was eluted to the clear tube by 5 ml of E6 elution buffer and then precipitated by the addition of 3.5 ml of isopropanol. Content was mixed and centrifuged 30 minutes at 4 °C. DNA pellet was washed with 70 % EtOH after the supernatant was threw away, centrifuged again 12 000 g for 3 minutes and after the EtOH removal left complete dry. Pellet was resuspended in 200 µl of miliQH_2O.

Concentration of isolated plasmid DNA was measured by the spectrophotometer *NanoDrop ND1000.*

Samples were stored at -20 °C.

3.2.3.1.4 Purification of plasmid DNA

Material: QIAprep®Spin Miniprep Kit 250 (*Qiagen*)

Equipment: centrifuge *Eppendorf MiniSpin plus®*, spectrophotometer *NanoDrop ND1000*

Method:

5 volumes of PB buffer was added to 1 volume of the DNA solution, mixed and then the solution was loaded into the column. Column was then centrifuged 10 000 g for 30 seconds, washed 2 times by 750 µl of PE buffer and centrifuged for 1 minute, 10 000 g. Flow-through liquid was discarded and the empty column was centrifuged for another 1 minute at 10 000 g to remove residual washing buffer. After that, the column was placed into clean tube and 20 µl of dH_2O was added. After 1 minute waiting, the tube with column was centrifuged for another 1 minute.

Concentration of purified plasmid DNA was measured by the spectrophotometer *NanoDrop ND1000*

Samples were stored at -20 °C.

3.2.3.2 Sequencing

Material: competent cells - bacterial strain *Escherichia coli* TOP10 (*Invitrogen),* bacterial LB medium– LB Broth (*Sigma-Aldrich),* plasmid pCR®-BluntII-TOPO® or pT7-gRNA, EcoRI restriction enzyme (*New England Biolabs*), restriction buffer (*New England Biolabs*), miliQH_2O

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, UV-transilluminator *UviTec Cambridge, BioTech*

Method:

Sequencing was utilized for analysis of target regions in *Platynereis pax2/5/8,* during the WMISH probes generation and for check of the right CRISPR oligonucleotide insertions into the expression vector for the guideRNAs synthesis.

For analysis of target regions in *Platynereis pax2/5/8,* these regions were amplified using the PCR reaction listed under "PCR analysis of the *pax2/5/8* target regions" and the resulting amplicons were cloned into the pCR®-BluntII-TOPO® plasmid. Plasmid DNA was amplified in E.coli TOP10 and isolated via the miniprep procedure, insertion was checked using the EcoRI restriction and the following reaction mix.

Restriction reaction mix:

plasmid DNA (pCR®-BluntII-TOPO®)	5 µl
restriction buffer	2 µl
restriction enzyme EcoRI (20 U/ml)	0.5 µl
miliQH_2O	12.5 µl

Restriction reaction was performed at 37 °C for 1 hour. Products were visualized and analyzed using the horizontal gel electrophoresisand 2% gel, run for 30 minutes at 110 V. Products were visualized using the UV-transilluminator. Samples with verified insertions were sent for sequencing in a following mixture. Same mixture was used for the sequencing of guideRNAs inserted in the pT7-gRNA vector.

Mix for sequencing:

DNA sample	2 µl (conc. about 200 ng/ul)
primer M13 (reverse)	1 µl
miliQH_2O	7 µl

Sequencing was provided by the *GATC-Biotech*.

3.2.3.3 Probe design for the whole mount RNA in-situ hybridisation (WMISH)

Material: primer oligonucleotides (*Sigma-Aldrich)*, cDNA isolated from 48 hpf old *Platynereis* embryos, AccuPrime Pfx SuperMix (Invitrogen), pCR®-BluntII-TOPO® plasmid, competent cells - bacterial strain *Escherichia coli* TOP10 (*Invitrogen),* restriction enzymes HindIII and EcoRV (*New England Biolabs),* restriction enzymes buffers (*New England Biolabs),* DIG RNA labelling mix (*Roche*), T7 RNA polymerase or SP6 RNA polymerase (*Roche*), miliQH_2O, agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*), QIAEX® Gel extraction Kit (*Qiagen*), QIAquick® Gel Extraction Kit (*Qiagen*)

Solutions: 1x TAE buffer

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, thermostat TCH 100 *Laboratorni pristroje Praha,* UV-transilluminator *UviTec Cambridge, BioTech*

Method:

Regions of WMISH probes for *pax2/5/8, otx* and *six3* were amplified by the PCR reaction using following reaction mix and PCR program. Primers were picked by the Primer3 software against the mRNA sequences from the NCBI database. As a template DNA was used cDNA isolated from the 48 hpf old *Platynereis* embryos.

NCBI database mRNA sequences:
pax2/5/8 = gi|440572046|gb|KC109637.1|
otx = gi|11877289|emb|AJ278856.1|
six3 = gi|202957445|emb|FM210809.1|

Reaction mix:

AccuPrime Pfx SuperMix	23.5 µl
primers	1 µl
cDNA	0.5 µl

PCR program:

95 °C	5 min
95 °C	*15 s*
55-65 °C	*30 s*
68 °C	*1 min*
	35x
72 °C	10 s
12 °C	„forever“

PCR products were loaded on the 0.8% agarose gel and run for 30 minutes at 110 V. Isolation of the DNA fragment was done by cutting off the band from the agarose gel and the DNA fragment was subsequently purified using QIAEX® Gel extraction Kit. 750 µl of the QC buffer was added with 10 µl of DNA-binding beads to the cut piece of the agarose gel and the agarose was dissolved at 50 °C. Solution was centrifuged for 1 minute at 10 000 g, supernatant was discarded and another 500 µl of the QC buffer was added. DNA bounded on the beads was purified using the wash in 300 µl of the PE buffer. Pellet was re-suspended in the 300 µl of the PE buffer, vortexed and centrifuged 10 000 g, 2 times. After the washing steps, solution was discard and pellet was let dry at 50 °C until it changed the colour from the transparent to the white. To the dry pellet were added 20 µl of miliQH_2O for the DNA dissolve. Purified DNA fragments were ligated into the pCR®-BluntII-TOPO® plasmid, which was subsequently transformed into the *E.coli* for amplification. Amplified plasmids were isolated using miniprep procedure and sequenced for the determination of the oligonucleotide insert orientation and thus proper selection of the restriction enzyme for the plasmid linearization before the RNA probe synthesis. 20 µg of each plasmid was linearized, *pax2/5/8* and *otx* plasmids were linearized by the HinDIII (20 U/ml), *six3* by the EcoRV (20 U/ml) restriction enzyme.

Plasmid linearization reaction mix:

DNA	20 µg
restriction enzyme	2 µl
restriction buffer	8 µl
miliQH_2O	adjust the volume of the reaction to 80 µl

Reactions were performed at 37 °C for 1 hour.

Products were purified to obtain RNAse free conditions using QIAquick® Gel Extraction Kit, resulting into the DNA concentration *pax2/5/8* = 200 ng/µl , *otx* = 225 ng/µl , *six3* – 260 ng/µl. 5 volumes of the PB buffer were added to 1 volume of the DNA solution and the mix was loaded to the plastic column. The column was spin for 30 seconds at 10 000 g, 750 µl of PE buffer was added and column was spin again for 60 seconds. The flow through was discard, empty column was centrifuged once again for 60 seconds, 10 000 g and the DNA was eluted by 50 µl of miliQH_2O to the clean eppendorf tube.

Approximately 10 µg of linearized plasmid was used for the *in-vitro* transcriptin for RNA probe synthesis. Reaction were mixed according to he following table.

In-vitro transcription reaction mix:

	pax2/5/8	*otx*	*six3*
linearized plasmid	5 µl	4.5 µl	3.9 µl
DIG RNA labelling mix	2 µl	2 µl	2 µl
buffer	2 µl	2 µl	2 µl
miliQH_2O	9 µl	9.5	10.1 µl
RNA polymerase	2 µl T7 RNA pol.	2 µl T7 RNA pol.	2 µl SP6 RNA pol.

Reaction were performed at 37 °C for 2 hours.

Quality of synthetized RNAs probes was checked using horizontal gel agarose electrophoresis using 2% agarose gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 15 minutes at 130 V. Products were visualized using the UV-transilluminator.

Probes for the pax6 and r-ops used from (Arendt et al. 2002)

3.2.3.4 Fixation of samples for the whole mount RNA in-situ hybridisation (WMISH)

Material: *Platynereis* embryos, MetOH

Solutions: 4 % PFA, PTw

Equipment: cooling incubator (*Q-Cell*)

Method:

Platynereis larvae were raised at 18 °C until they reached the appropriate stage (24, 48 or 72 hpf). Fixative solution of 4 % PFA in PTw was prepared in advance from 16 % PFA stock and chilled on ice. Larvae were fixated for 2 hours at room temperature in 1.5 ml eppendorf tubes, then washed 3 times for 5 minutes in PTw and another 2 times in ice cold MetOH. After the fixation procedure, larvae were stored at -20 °C.

3.2.3.5 Whole mount RNA in-situ hybridisation (WMISH)

Material: MetOH (*Penta*), proteinase K recombinant PCR grade (*Roche),* anti-DIG/AP (*Roche*), anti-acetylated α-tubulin antibody T6793 (*Sigma-Aldrich*), Alexa Fluor®555 antibody (*A-21422*) Goat anti-Mouse IgG (*Life Technologies*), synthetized WMISH probes, 4 or 24-well plates (*TPP*), NBT/BCIP substrate (*Roche*) or Vector® blue substrate Kit (*Vector laboratories*)

Solutions: PTw, 60 % MetOH, 30 % MetOH, 2 mg/ml glycine in PTw, 1 % Triethanolamine, 4 % PFA, 50% FA + 2x SSCT, 2x SSCT, 0.2x SSCT, AP+ solution, AP- solution, TRIS buffer

Equipment: thermal block *THERMO BLOCK TDB-120 Biosan,* rocking table *Kavalier LT2*

Method:

Unless stated otherwise, all solutions were kept on ice during the first day of procedure.

During the first day of whole WMISH procedure, embryos were transferred from MetOH to PTw, through rehydratation 500 µl wash steps in 60% MetOH in PTw, 30% MetOH and PTw 4 times. Each larvae stage was kept in separate eppendorf tube. After the rehydratation, larvae were digested with 0.1 mg/ µl proteinase K, 30 seconds for 24 hpf old and 1 minute for 48 and 72 hpf old animals. Digestion was stopped by 2 mg/ml glycine in PTw by washing 2 times for 2 minutes and one subsequent wash in PTw. To improve in-situ results, samples were then washed in 1 % Triethanolamine in PTw for 5 minutes and 2 times in PTw after that. As a next step, samples were refixated in 4 % PFA for 30 minutes at room temperature, washed 5 times in PTW and incubated in 100 µl of hybe buffer at RT for 10 minutes and 1.5 hours at 63 °C. 50 µl of each probe in hybridisation buffer was added to the samples at 63 °C in concentration 2 ng/ µl after their denaturisation at 85 °C for 10 minutes. Hybridization was performed for 20 hours.

Probes were removed on the second day of the procedure and samples were washed in 1x SSCT for 15 minutes, 2x 30 minutes in 50% FA + 2x SSCT, 2x SSCT for 15 minutes and 2x 30 minutes in 0.2x SSCT,

all at 63 °C. Afterwards one wash in PTw for 30 minutes was performed, followed by incubation in 150 µl of blocking buffer for 30 minutes, both at room temperature. After that, samples were incubated overnight, rocking at 4 °C with 200 µl of anti-DIG/AP diluted 1:4000 and with acetylated α-tubulin T6793 antibody diluted 1:500.

Third day is consisting of washing steps, first 3x in PTw for 5 minutes followed with 4x in PTw for minutes, all at room temperature, rocking. These washing steps were followed by overnight PTw wash at 4 °C, rocking.

On the fourth day the signal was developed. In case of NBT/BCIP substrate, initial washing was done in AP- 2x for 5 minutes, followed washing in AP+ 2x for 5 minutes. After the second was, embryos were transferred to 4-well (24-well) plate and 400 µl of the developing solution consisting of NBT/BCIP each 3.3 µl per 1 ml of AP+ was added to the samples. Staining was stopped by washing 5x in PTw at 4 °C. For the vector blue substrate, the washing steps were performed in TRIS buffer. Incubation with anti-acetylated tubulin secondary antibody Alexa Fluor®555 diluted 1:500 in hybridization buffer for 1 hour was performed after the washing steps. Incubation was stopped by washing in PTw, 5 times for 5 minutes.

3.2.4 Generation of the *pax2/5/8 Platynereis* knockout line

Introduction of the CRISPR/Cas9 technology to the *Platynereis* was implemented to the generation of a functional tool to perform the *pax2/5/8* knockout. Targeted regions were analysed using the PCR analysis followed by sequencing and 4 guideRNAs expression constructs were prepared. 4 guideRNAs were produced and subsequently injected together with the Cas9 mRNA to the one-cell stage Platynereis embryos. F0 injected generation was analysed using the genotyping assay and the T7-Endonuclease assay.

3.2.4.1 PCR analysis of the *pax2/5/8* target regions

Material: primer oligonucleotides (*Sigma-Aldrich*), DreamTag Green PCR MasterMix (*Thermo Scientific*), agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*), miliQH_2O

Solutions: 1x TAE buffer

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, UV-transilluminator *UviTec Cambridge, BioTech*

Method:

PCR reactions were performed using the following reaction mix and PCR program. Primers were designed against the *in-silico* predicted sequence of *Platynereis pax2/5/8* and used primer combinations were ID1 + ID2 for the analysis of the pax2/5/8 1st exon, ID27 + ID28 for the analysis of the pax2/5/8 2nd exon. Combination ID1 + ID28 was used for the analysis of excisions in the intron region followed by subsequent ligation.

Reaction mix:

DreamTag Green MasterMix	12.5 µl
miliQH_2O	6.5 µl
primers	1 µl
DNA	5 µl

PCR program:

95 °C	1 min
95 °C	*30 s*
60 °C	*30 s*
72 °C	*1 min*
	35x
72 °C	5 min
12 °C	„forever“

PCR products were visualized and analysed using horizontal gel agarose electrophoresis using 2% agarose gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 45 minutes at 110 V. Products were visualized using the UV-transilluminator.

3.2.4.2 Generation of guideRNA expression constructs

One guideRNA for each target site was synthetized out of customized pT7-gRNA vector. Synthetized CRISPR oligonucleotides matching to the target sites of *Platynereis pax2/5/8* 1st and 2nd exon were annealed together, resulting into the double strand fragments of DNA. These fragments were subsequently ligated into the BsmBI linearized pT7-gRNA, amplified in TOP10 E.coli and isolated by the midiprep procedure. Insertion of the CRISPR oligonucleotides into the vector was checked by the PCR, using as primers one of the CRISPR oligonucleotides together with the M13 primer located on the vector backbone. Subsequent check of the oligonucleotides insertion was provided by sequencing. Vector for each guideRNA was linearized using BamHI cut and purified to obtain the RNase free conditions. 4 guideRNAs were synthetized, two for each exon of the *Platynereis pax2/5/8*.

3.2.4.2.1 CRISPR oligonucleotides annealing

Material: *Sigma-Aldrich* synthesized CRISPR oligonucleotides

Equipment: thermal cycler *T100™ BIO-RAD*

Method:

Matching CRISPR oligonucleotides for the guideRNA production were annealed together using the following program. 5 µl of each synthesized oligonucleotide used.

CRISPR oligonucleotide matching pairs:

pax2/5/8 1st exon forward CRISPR = ID5 + ID6
pax2/5/8 1st exon reverse CRISPR = ID34A + ID34B
pax2/5/8 2nd exon forward CRISPR = ID30A + ID30B
pax2/5/8 2nd exon reverse CRISPR = ID35A + ID35B

Annealing program:

denaturation	95 °C	3 minutes
annealing and cooling	85 °C	30 seconds
	-1 °C/cycle up to 25 °C	
final cooling	25 °C	"forever"

3.2.4.2.2 pT7-gRNA vector preparation

Material: pT7-gRNA plasmid, restriction buffer 3.1 (*New England Biolabs*), restriction enzyme BsmBI (*New England Biolabs*) miliQH_2O

Equipment: thermal cycler *T100™ BIO-RAD*

Method:

pT7gRNA plasmid was restricted using the BsmBI restriction enzyme before the CRISPR oligonucleotides insertion. Restriction reaction mixtures were prepared according to the following table and the reaction was performed at 55 °C for 1.5 hour.

Restriction reaction mix:

plasmid DNA (pT7-gRNA)	15 µl
buffer	5 µl
restriction enzyme BsmBI (10 U/ml)	2 µl
miliQH_2O	28 µl

3.2.4.2.3 Ligation of CRISPR oligonucleotides into the pT7-gRNA vector

Material: quick T4 DNA ligase enzyme (*New England Biolabs)*, ligation buffer (*New England Biolabs*), miliQH_2O, BsMBI linearized vector pT7-gRNA, annealed CRISPR oligonucleotides for insertion, competent cells - bacterial strain *Escherichia coli* TOP10

Method:

5 µl of ligation mixture prepared according to the following table was used for transformation of 40 µl of TOP10 E.coli competent cells. For the isolation of the plasmid DNA was used midiprep procedure.

Ligation reaction mix:

target vector pT7-gRNA, linearized	50 ng (1 µl)
3 fold molar excess of insert	3 µl
miliQH_2O	adjust volume to 10 µl (vector + insert + H_2O)
quick T4 DNA ligase	1 µl
quick T4 buffer	10 µl

3.2.4.2.4 Check of CRISPR oligonucleotides insertion into the pT7-gRNA vector

Material: pT7-gRNA plasmid with CRISPR oligonucleotides inserted, DreamTag Green PCR MasterMix (*Thermo Scientific*), agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*), miliQH_2O

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300

Method:

Insertion of CRISPR oligonucleotides into the pT7-gRNA was checked by the PCR reaction using primer pairs consisting of a CRISPR oligonucleotide used as forward primer and M13 reverse primer. Reaction mixture was prepared according to the following table.

Reaction mix:

DreamTag Green MasterMix	12.5 µl
miliQH_2O	6.5 µl
primers	1 µl
DNA	5 µl

PCR program:

95 °C	1 min
95 °C	*30 s*
60 °C	*30 s*
72 °C	*1 min*
	35x
72 °C	5 min
12 °C	„forever“

Resulting PCR products were visualized and analysed using horizontal gel agarose electrophoresis using 2% gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating

in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 30 minutes at 110 V.

Subsequent check of oligonucleotides insertion was done by sequencing provided by *GATC Biotech*.

3.2.4.2.5 Linearization of the pT7-gRNA vector before guideRNA synthesis

Material: pT7-gRNA plasmid, restriction buffer, restriction enzymes BamHI (*New England Biolabs*) miliQH_2O

Equipment: thermal cycler *T100™ BIO-RAD*, spectrophotometer *NanoDrop ND1000*

Method:

Restriction reaction mixtures were prepared according to the following table:

plasmid DNA (pT7-gRNA)	10 µg
buffer	8 µl
restriction enzyme BamHI (20 U/ml)	2 µl
miliQH_2O	80 µl

Reaction was performed at 37 °C for 1.5 hour.

3.2.4.2.6 Purification of the pT7-gRNA plasmid DNA

Material: QIAprep®Spin Miniprep Kit 250 (*Qiagen*)

Equipment: centrifuge *Eppendorf MiniSpin plus®*, spectrophotometer *NanoDrop ND1000*

For the method description see **Purification of plasmid DNA**

3.2.4.3 *In-vitro* transcription - guideRNA synthesis

Material: MegaShortScript™Kit (*Invitrogen*), agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*), miliQH_2O

Solutions: 1x TAE buffer

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, UV-transilluminator *UviTec Cambridge, BioTech,* fluorometer *Quibit™ Invitrogen*

Method:

Reactions were prepared according to following table.

reaction buffer	2 µl
ATP	2 µl
CTP	2 µl
GTP	2 µl
UTP	2 µl
template DNA (linearized plasmid)	less than 8 µl
T7 polymerase mix	2 µl
miliQH_2O	adjust volume to 20 µl

Reaction synthesising the CRISPR guideRNAs was performed at 37 °C for 6 hours.

Quality of synthetized guideRNAs was checked using horizontal gel agarose electrophoresis using 2% agarose gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 15 minutes at 130 V.

Sample concentrations were measured by Quibit fluorometer, using Quant-iT™ RNA Assay Kit. Samples were stored at -80 °C in aliquots.

3.2.4.4 Microinjection

Material: one-cell stage *Platynereis* embryos, natural sea water, agarose plates (2% agarose (*Serva*) in NSW), borosilicate glass capillaries, injection mix

Equipment: injection set-up – pressure injector FemtoJet (*Eppendorf*), micromanipulator TransferMan NK (*Eppendorf)*, inverted microscope *Eclipse TS100 with ELWD 0.3 objective selector (Nikon)*

Solutions: 70 µl of protK in 30 ml NSW, injection mix

Injection mix:

guideRNA1	10 ng/µl
guideRNA2	10 ng/µl
guideRNA3	10 ng/µ
guideRNA4	10 ng/µ
Cas9 mRNA	300 ng/µl
phenolRed	6 µl

Samples were stored at -80 °C in aliquots.

Cas9 mRNA obtained from Z. Kozmik, synthetized out of plasmid MLM3613 (addgene #42251), insert: *Streptococcus pyogenes* Cas9

Method:

The injection mix of gRNAs and Cas9 mRNA was co-injected directly into one-cell stage embryos with phenolRed used as co-injection marker. Embryos were obtained from the wild-type cross

of animals from breeding culture of Department of Transcriptional Regulation (Institute of Molecular Genetics of the ASCR, v. v. i.). Male and female worms close to maturity were separately placed into two different tanks with NSW and moved together as fully matured animals on the morning of the day of injection. 45 minutes after the spawning, embryos were removed from the own-produced jelly by NSW washing in the sifter. Embryonic surface was softened to prevent the capillary sliding by washing the embryos in sifter by the solution of proteinase K for 25 seconds. After the proteinase K treatment, embryos were transferred onto the agarose plate (prepared in advance) and the plate was placed under the microscope. Injection mix was stored on ice during the handling and centrifuged 4 000 g for 1 minute before the injection, to avoid the clog of the capillary. After the centrifugation, 1 µl of injection mix was loaded into the capillary and the capillary was placed in capillary holder of micromanipulator. Embryos were injected with the injection mix one after the other. Injected volume into the embryo did not exceed 1/10 of the cell´s volume. Injected embryos were subsequently collected and left develop at 18 °C.

3.2.4.5 Genotyping assay of F0 injected embryos

Material: 10x PCR buffer DyNAzyme (*Finnzymes*), miliQH_2O, primers (*Sigma-Aldrich*), DreamTag Green PCR MasterMix (*Thermo Scientific*), agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker GeneRuler DNA ladder Mix (*Thermo Scientific*)

Solutions: proteinase K recombinant PCR grade (*Roche),* 1x TAE buffer

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, UV-transilluminator *UviTec Cambridge, BioTech*

Method:

F0 generation of injected animals was analysed using genotyping assay consisting of multiple PCR reactions using the DNA from injected animals. To isolate the DNA from the injected embryos lysed 48 hpf was used following lysation mix.

Lysation mix:

miliQH_2O	43 µl
10x PCR buffer DyNAzyme	5 µl
proteinase K	2 µl

Embryos were put into the PCR plastic tubes with the lysation mix and the reaction was run in the thermocycler according to the following program.

Lysation program:

65 °C 5 hours
80 °C 25 minutes
12 °C "forever"

Samples of isolated DNA were stored at 4 °C.

Targeted genomic regions were amplified using PCR reaction performed in thermocycler using the following reaction mix and PCR program. Used primer combinations were ID1 + ID2 for the analysis of the pax2/5/8 1st exon, ID27 + ID28 for the analysis of the pax2/5/8 2nd exon. Combination ID1 + ID28 was used for the analysis of excisions in the intron region followed by subsequent ligation. As a positive control of DNA presence was used genotyping for α-tubulin using the primer combination ID36 + ID37.

Reaction mix:

DreamTag Green MasterMix	12.5 µl
miliQH_2O	6.5 µl
primers	1 µl
DNA	5 µl

PCR program:

95 °C 1 min
95 °C 30 s
60 °C 30 s
72 °C 1 min
35x
72 °C 5 min
12 °C „forever"

Resulting PCR products were visualized and analysed using horizontal gel agarose electrophoresis using 3% gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 45 minutes at 110 V. Products were visualized using the UV-transilluminator. Mutations can be detected as additional bands compared to wild-type DNA used as a control in case of primer combinations ID1 + ID2 and ID27 + ID28 analysing the 1st and the 2nd exon of *Platynereis pax2/5/8*. For primer combination ID1 + ID28, mutation can be detected as a band presence, since in case of wild-type, no PCR band is obtained. This could be possibly because too long sequence between these two primers in case of wild-type DNA, which could suggest the intron region present compared shortened region due to mutation in F0 animal.

3.2.4.6 T7 endonuclease assay of F0 injected animals

Material: 10x PCR buffer DyNAzyme (*Finnzymes*), miliQH_2O, primers (*Sigma-Aldrich*), DreamTag Green PCR MasterMix (*Thermo Scientific*), agarose (*Serva*), ethidium bromide (*Sigma-Aldrich*), marker

GeneRuler DNA ladder Mix (*Thermo Scientific*), NEB buffer 2 (*New England Biolabs*), T7 endonuclease (*New England Biolabs*)

Solutions: 1x TAE buffer

Equipment: thermal cycler *T100™ BIO-RAD,* horizontal electrophoresis set-up *BIO-RAD,* horizontal electrophoresis electrical source *BIO-RAD* PowerPac™300, UV-transilluminator *UviTec Cambridge, BioTech*

Method:

F0 generation of injected animals was analysed using T7 endonuclease assay consisting of multiple PCR reactions using the DNA from mixed population of injected animals. Each sample, analysed by T7 endonuclease reaction was consisting of DNA from 5 injected *Platynereis* embryos. To isolate the DNA from the injected embryos lysed 48 hpf was used following lysation mix.

Lysation mix:

miliQH_2O	43 µl
10x PCR buffer DyNAzyme	5 µl
proteinase K	2 µl

Embryos were put into the PCR plastic tubes with the lysation mix and the reaction was run in the thermocycler according to the following program.

Lysation program:

65 °C 5 hours
80 °C 25 minutes
12 °C "forever"

Samples of isolated DNA were stored at 4 °C.

Targeted genomic regions were amplified using PCR reaction using the following reaction mix and PCR program. Used primer combinations were ID1 + ID2 for the analysis of the pax2/5/8 1st exon, ID27 + ID28 for the analysis of the pax2/5/8 2nd exon.

Reaction mix:

DreamTag Green MasterMix	10 µl
miliQH_2O	6.5 µl
primers	1 µl
DNA	2.5 µl

PCR program:
95 °C 1 min
95 °C 30 s
60 °C 30 s
72 °C 1 min
35x
72 °C 5 min
12 °C „forever“
2.5 µl of NEB buffer 2 were added to the each sample and PCR amplicons were denatured and slowly reannealed to facilitate the heteroduplex formation according to the following program.

Denaturation re-annealing program:

denaturation	95 °C	3 minutes
annealing and cooling	85 °C	30 seconds
	-1 °C/cycle	
final cooling	25 °C	“forever”

After the program proceeded, following reaction was performed at 37 °C for 1 hour.

Restriction mix:

NEB buffer “2”	3 µl
T7 enzyme	0.5 µl/1 sample
miliQH_2O	adjust volume to 30 µl

Result products after the T7 endonuclease restriction reaction were visualized and analysed using horizontal gel agarose electrophoresis using 3 % agarose gel. Agarose gel was prepared from agarose powder diluted in the TAE buffer by heating in microwave oven and to the liquid was added EtBr (5 µl for 50 ml of gel). Samples were loaded after the gel solidification and running for 45 minutes at 110 V. Products were visualized using the UV-transilluminator. Single nucleotide mutations were detected as additional bands compared to wild-type DNA used as a control. As a negative was used PCR mix without the DNA added.

3.2.5 Used software

Adobe Illustrator CS6 - graphic editor used for the figures and schemes drawing
Adobe Photoshop CS6 - image editor used for the photography processing
Adobe Photoshop Lightroom 4.4 - image editor used for the photography processing
FastStone Viewer v5.5 - image editor used for the photography processing
Fiji/ImageJ - image editor used for the photography processing, version 1.50i
Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) - on-line interface used for the primers design
phyloT http://phylot.biobyte.de/ - phylogeneitc tree generator, based on NCBI taxonomy, used for the visualisation of phylogenetic relations ship among annelida species

4. RESULTS

4.1 Expression of selected set of genes in *Platynereis* eyes in wild-type

Based on a previous study revealing the hypothetical scheme of the ancestral opsin regulation (Vopalensky 2009), we have predicted genes with potential role in the early eye and nervous system development in *Platynereis*. We selected *pax6, pax2/5/8, prox1, otx, six3, pax3/7, r-ops1* as genes with a potential role in eye development in *Platynereis.* RT-PCR (Fig. 10) was initially performed to confirm the expression of genes of interest during the *Platynereis* early development and to determine gene expression onsets.

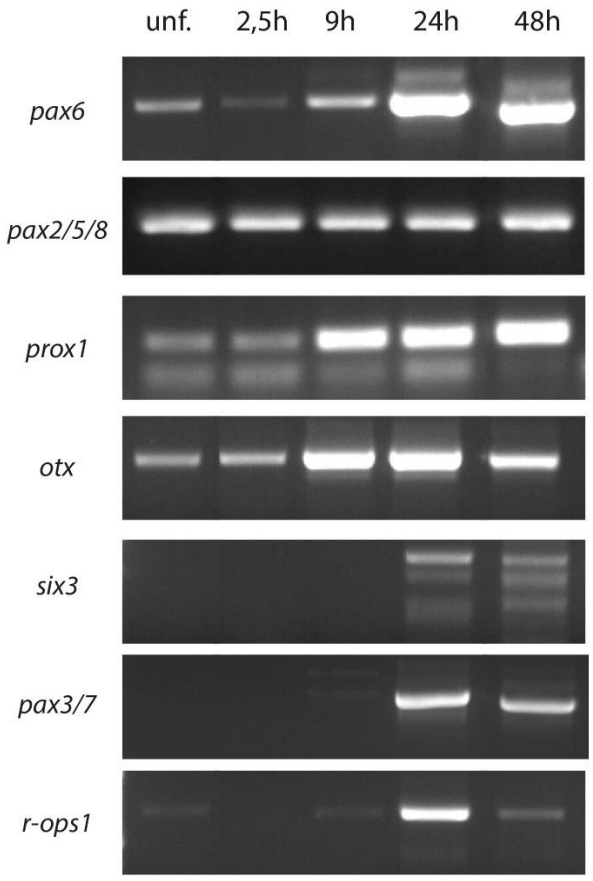

Fig. 10: RT-PCR of the *Platynereis pax6, pax2/5/8, prox1, otx, six3, pax3/7* and *r-ops1* genes performed on cDNA from unfertilized eggs, 2.5, 9, 24 and 48 hpf old larvae.

Expression of *pax6, pax2/5/8, prox1* and *otx* was detected in unfertilized eggs, followed by ongoing expression at 2.5, 9, 24 and 48 hpf. *pax3/7* and *r-ops1* are faintly expressed at 9 hpf, more robust expression was detected at 24 and further at 48 hpf. Expression of *six3* was detected at 24 and 48 hpf. Notable increase of expression of investigated genes at 24 hpf may be linked to the begin of larval eye development at this stage.

To confirm the results from RT-PCR, we set out to analyse expression of these genes on whole mounts at stages 24, 48 and 72 hpf (Fig. 11). Expression of all analysed genes in the larval or adult eyes of *Platynereis* and in different stages was confirmed by the WMISH. Expression of *pax6* and *six3* was detected only in larval eyes, expression of *r-ops1* was detected only in adult eyes. Expression of *pax2/5/8* and *otx* was detected in both types of eyes, which may indicate the involvement in visual process provided by larval and adult eyes as well.

Based on our results from RT-PCR and whole mounts, two early expressed *Platynereis* genes were selected for further research - *pax6,* which is expressed in the larval eyes and *pax2/5/8*, which is expressed in both larval and adult eyes.

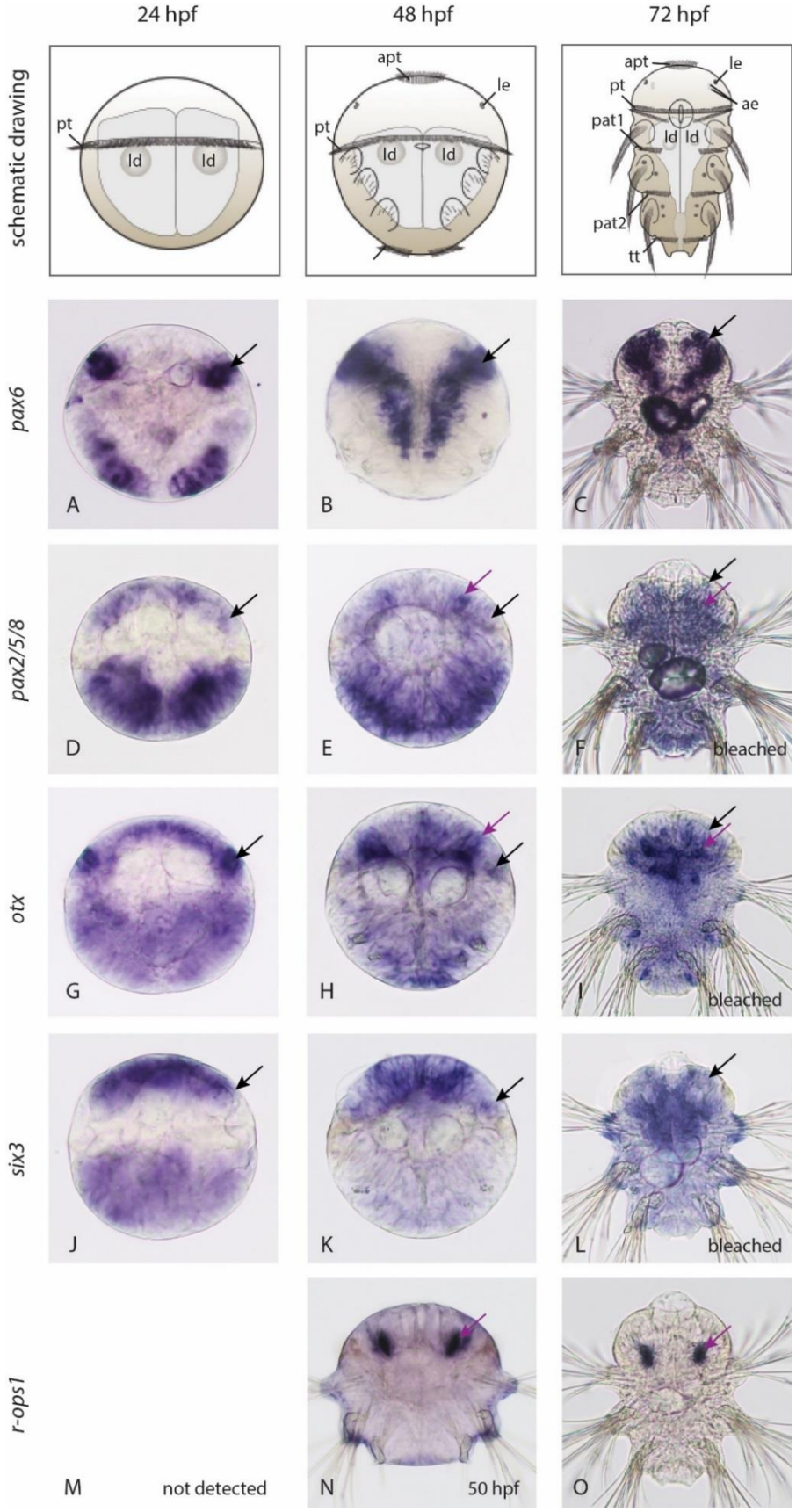

Fig. 11: Wild-type expression analysis of *pax6* (A-C), *pax2/5/8* (D-F), *otx* (G-H), *six3* (J-L) and *r-ops1* (M-O*)* at 24, 48 and 72 hpf old *Platynereis* larvae. *pax6, pax2/5/8, otx* and *six3* were detected in larval eyes; *pax2/5/8, otx3, six3* and *r-ops1* was detected in adult eyes. Larval and adult eyes are marked with black and purple arrows, respectively. WMISH signal of F, I and L animals were bleached by MetOH to reduce the background signal.

4.1.1 Expression analysis of *Platynereis pax6* in wild-type

Wild-type expression of the *pax6* in the larval eyes of *Platynereis* was confirmed by the WMISH procedure (see Fig. 11, pictures A-C).

4.1.2 Expression analysis of *Platynereis pax2/5/8* in wild-type

To establish the gene expression pattern of *pax2/5/8* in the *Platynereis* larvae the WMISH procedure was performed, using bright field light microscopy and confocal microscopy (Fig. 12 and Fig. 13). WMISH probe was designed using the *Platynereis pax2/5/8* mRNA (complete cds, GenBank: KC109637.1).

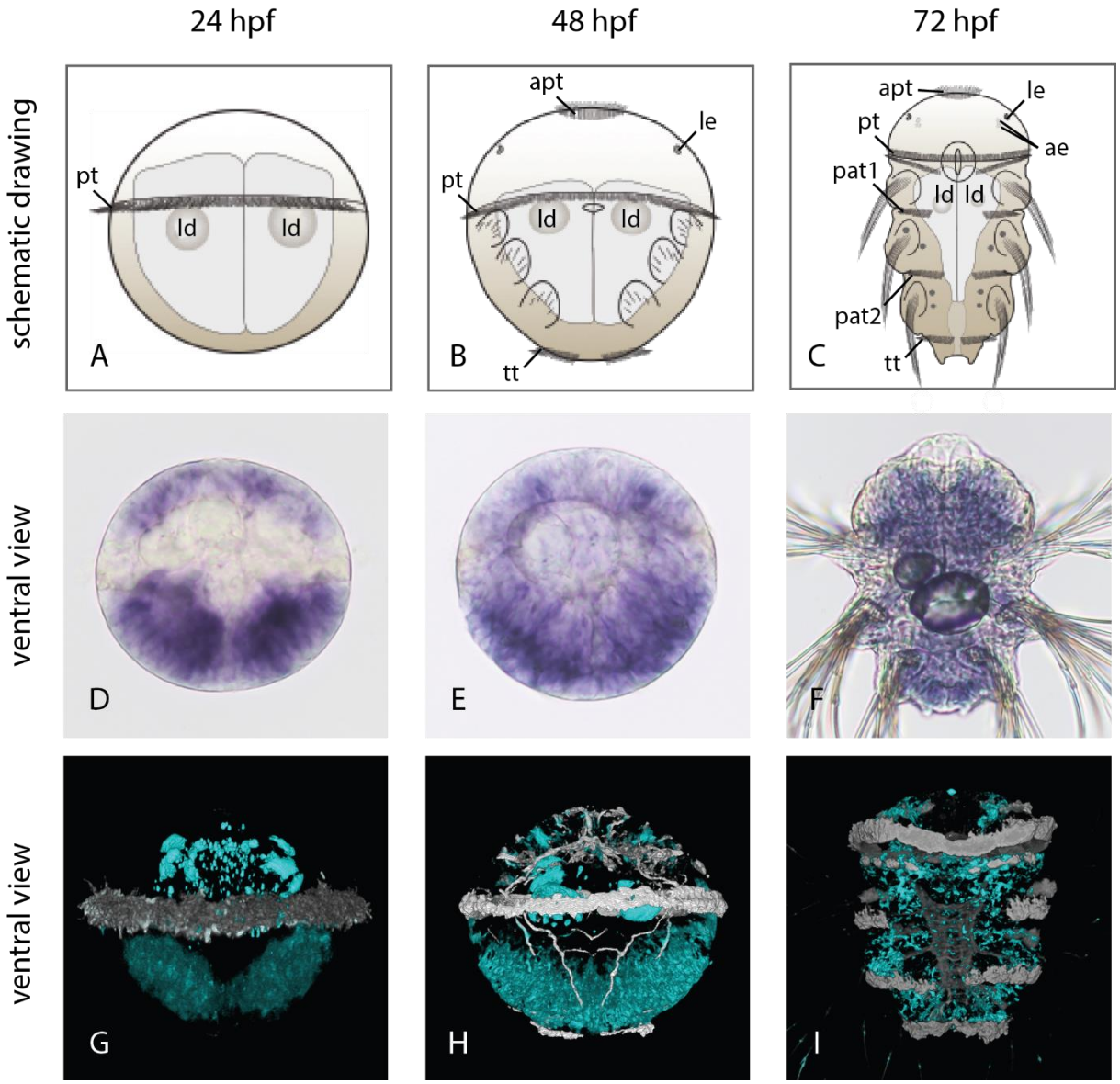

Fig. 12: *Platynereis dumerilii pax2/5/8* wild-type expression pattern at 24, 48 and 72 hpf, (A- C) - Schematic drawing of *Platynereis* embryos, (D-F) - bright field light microscopy, (G-I) - confocal microscopy 3D reconstruction , WMISH signal visible as purple, cyan = WMISH signal, grey = anti-acetylated tubulin antibody staining marking the ciliary structures and axonal scaffold.

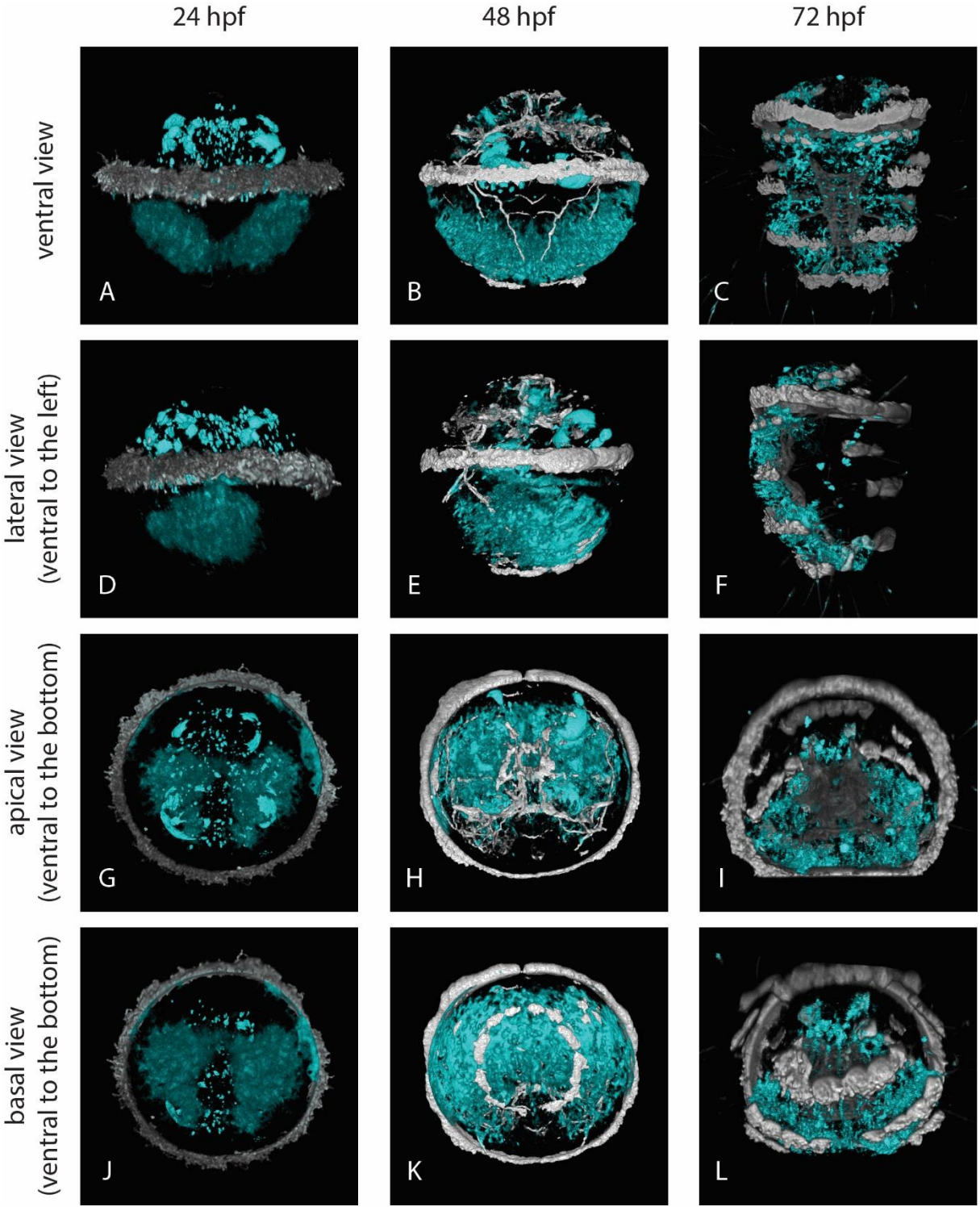

Fig. 13*: Platynereis pax2/5/8* wild-type expression pattern confocal microscopy 3D reconstruction, ventral view (A-C), lateral view (D-F), apical view (G-I) and basal view (J-L). Multiple views given to provide the 3D perspective, cyan = WMISH signal, grey = anti-acetylated tubulin antibody staining marking the ciliary structures and axonal scaffold.

4.2 Analysis of *Platynereis pax6* knockout line

To investigate the role of *pax6* in the eye development, a *Platynereis pax6* knockout line has previously been established in our lab using zinc finger nuclease technology (Z. Kozmik, unpublished data). Part of the RED domain (exon3) was successfully targeted resulting in non-functional truncated form of Pax6 protein. Moreover, the 61 nucleotides deletion is easily detectable by genotyping (Fig. 14).

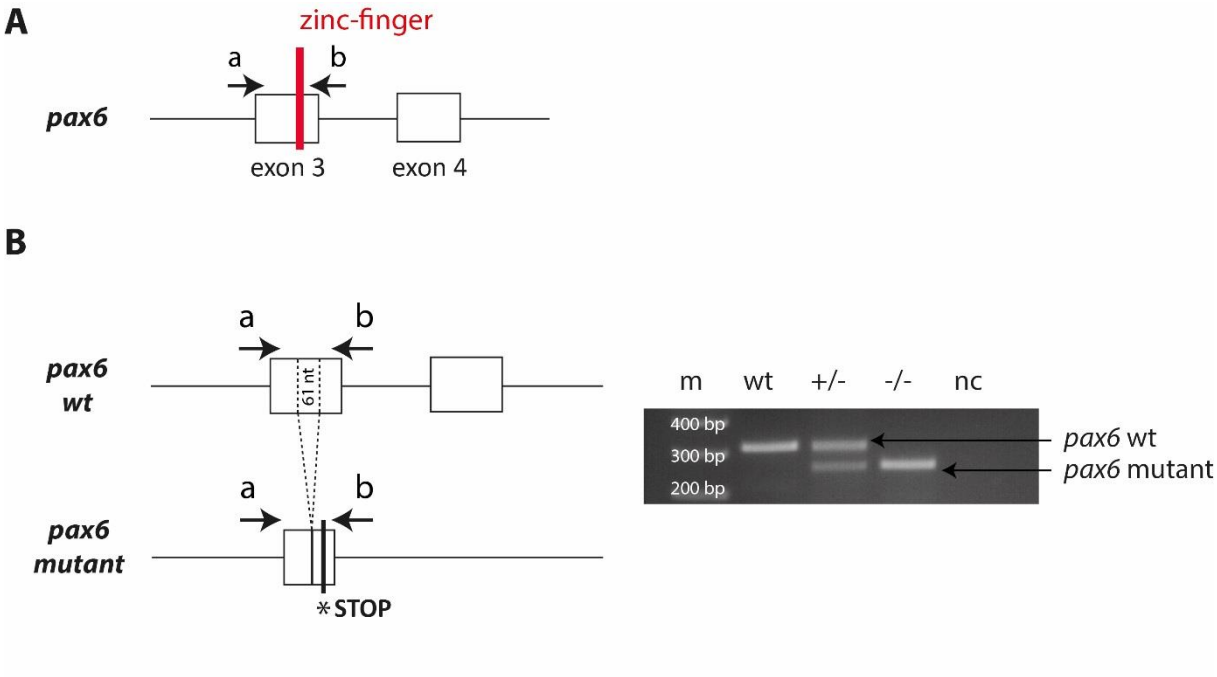

Fig. 14*: Platynereis pax6* mutant line scheme
A: Scheme of the targeted region. Zinc finger nuclease was targeted into the 3rd exon of the *Platynereis pax6*, which leads to the partial deletion of the RED domain and a premature stop-codon (marked with *). Non-functional truncated form of the Pax6 protein is encoded.
B: visualization of genotyping results from pax6 wt and mutant using primers "a" and "b" depicted in panel A.
a,b = genotyping primers, m = marker, wt = wild-type animal, +/- = heterozygote *pax6* mutant, -/- = homozygote *pax6* mutant, nc = negative control

4.2.1 Analysis of gene dosage effect in the *Platynereis pax6* knockout line

Platynereis pax6 knockout homozygote mutants are not able to survive more than approximately four weeks, however, heterozygotes are viable and able to reproduce (Z. Kozmik, unpublished data). We tested the number of *pax6* alleles present by WHISH and subsequent genotyping. Comparison of the *pax6* expression pattern in wild-type (wt) animals, *pax6* knockout heterozygotes and *pax6* knockout homozygotes implies the haplosufficiency of one *pax6* allele (Fig. 15). 20 animals with the WMISH signal present were tested (Fig. 15 - A and B), out of which seven animals were confirmed by genotyping as wt and 14 animals were confirmed as *pax6* heterozygote mutants. No *pax6* homozygote mutant was detected within the animals with the WMISH *pax6* signal present. 30 animals without the WMISH pax6 signal were tested, out of which eight *pax6* homozygote mutants were confirmed by genotyping (Fig. 15 - C).

No homozygote mutant was found among the tested animals with the *pax6* WMISH signal present, contrary to the repeated presence of mutants among blank (without the WMISH signal) animals.

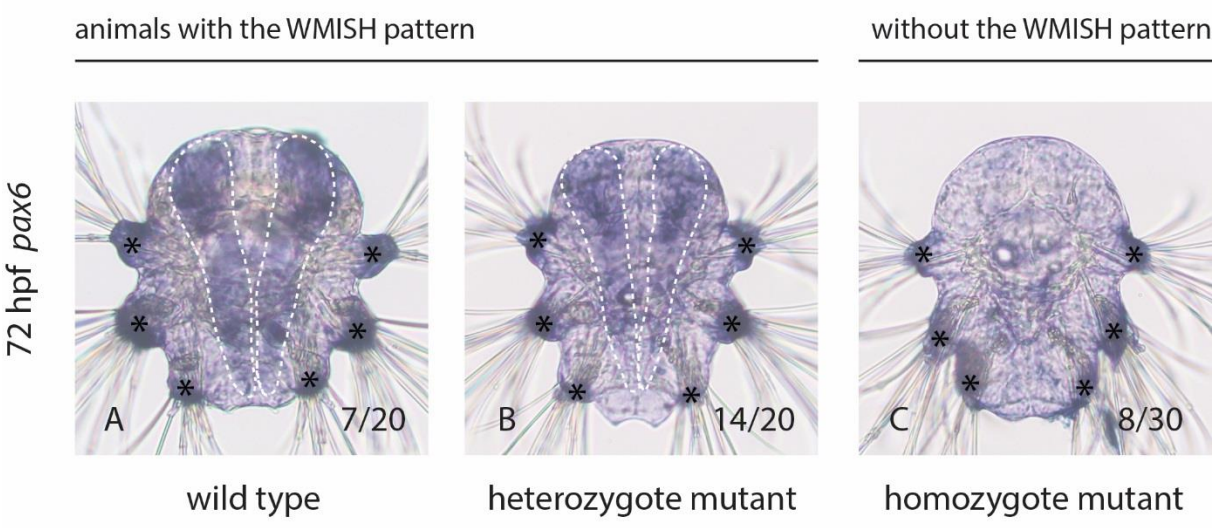

Fig. 15: Comparison of *pax6* expression pattern in wild type (A), *pax6* heterozygote (B) and *pax6* homozygote mutants (C). Area of pax6 expression pattern is marked by dashed line. To avoid false negative results due to the short period of the WMISH signal development, samples were overstained resulting into the non-specific staining in parapodia (marked by asterisks).

4.2.2 Analysis of selected genes in the *pax6* knockout *Platynereis*

Examination of the expression pattern changes of *pax2/5/8, otx, six3* and *r-ops1* between wt and mutant animals was selected as the initial step of the *Platynereis pax6* mutant line analysis.

Expression patterns of *pax2/5/8, otx, six3* and *r-ops1* genes visualized by WMISH was not affected by the *pax6* deletion in *Platynereis* (Fig. 16), suggesting that these genes are not regulated by *pax6*.

This is in contrast to multiple interactions of *pax6* with homologues of these genes observed in various organisms.

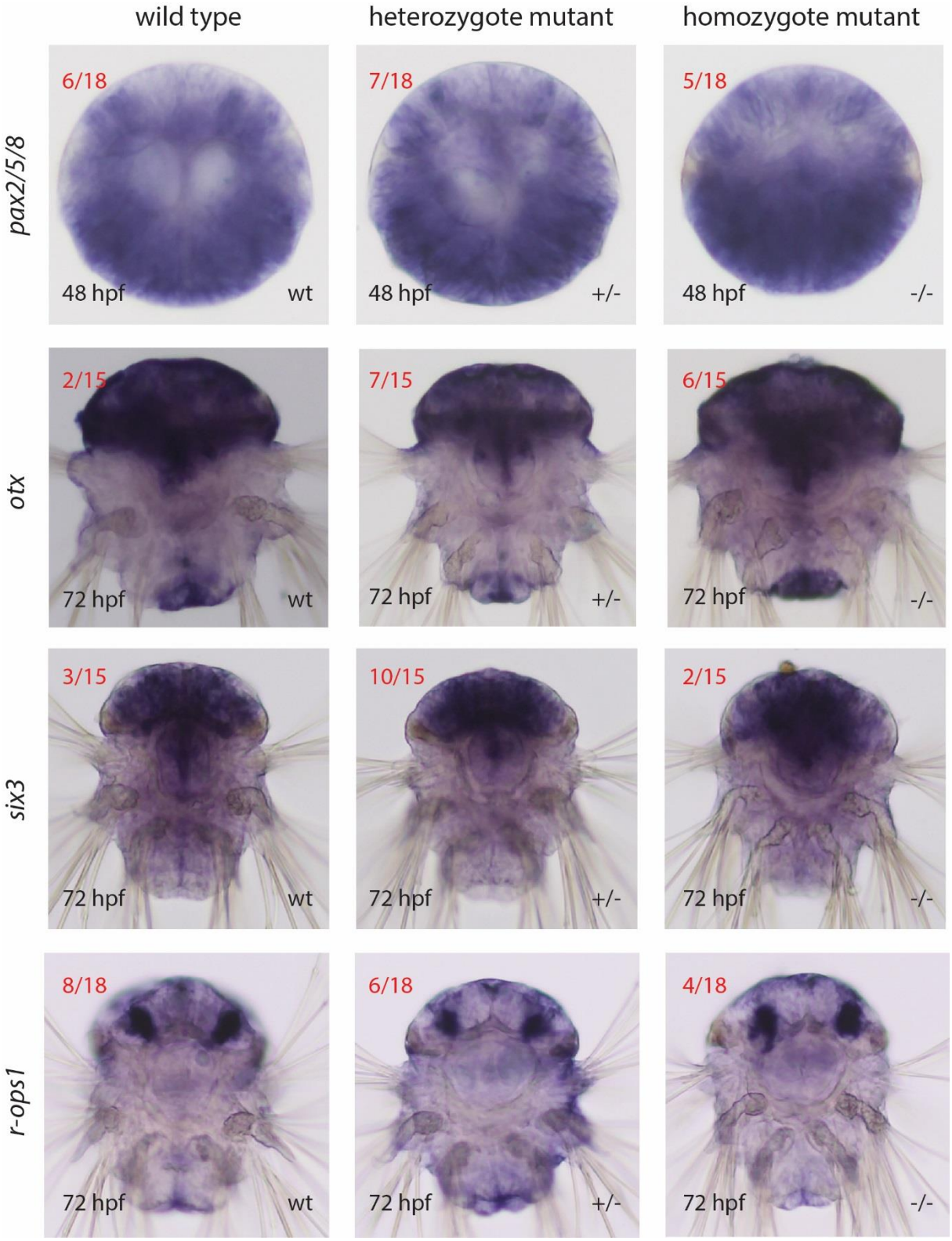

Fig. 16: Expression pattern of *pax2/5/8, otx, six3* and *r-ops1* in *pax6* wt, heterozygote and homozygote mutants. No changes were observed in the expression pattern in *pax6* wt, heterozygote mutants and homozygote mutants for all genes. Red numbers = number of given genotype/number of animals tested.

4.3 Generation of a *Platynereis pax2/5/8* knockout line

To further analyse the role of *pax* genes in eye development, we applied RNA-guided genome editing strategy using the CRISPR/Cas9 system to generate *pax2/5/8* knockout line. This approach allows a site-specific DNA double-stranded breaks *in-vivo*, resulting into the targeted genomic changes. Site specific cleavage provided by the Cas9 endonuclease is determined by the base-pairing complementarity of the designed guideRNA to the target site and by the presence of Protospacer Adjacent Motif (PAM). This motif consists of one nucleotide on the 3´ end of the guideRNA target sequence, directly followed by the GG dinucleotide and it is essential for the DNA cleavage (Jinek et al. 2012). The possibility to target multiple genomic loci simultaneously (Jao et al. 2013) renders this system a highly efficient mutagenesis tool. Results obtained by the Cas9 induced mutagenesis in *Platynereis* confirmed the ability of the used system to specifically introduce mutations into the *Platynereis pax2/5/8.*

4.3.1 Gene organization analysis in *Platynereis*

The fact that the *Platynereis dumerilii* genome is not currently available and that no other *Platynereis pax2/5/8* records exist, except for mRNA sequence in NCBI (accession number KC109637.1), led us to a comparative analysis with the genomes of two related species, namely *Capitella teleta* and *Helobdella robusta* (Fig. 17 A), in an attempt to infer the putative *Platynereis pax2/5/8* exon-intron organization. Consequently, genotyping primers were prepared (for illustration see scheme in Fig. 17 B).

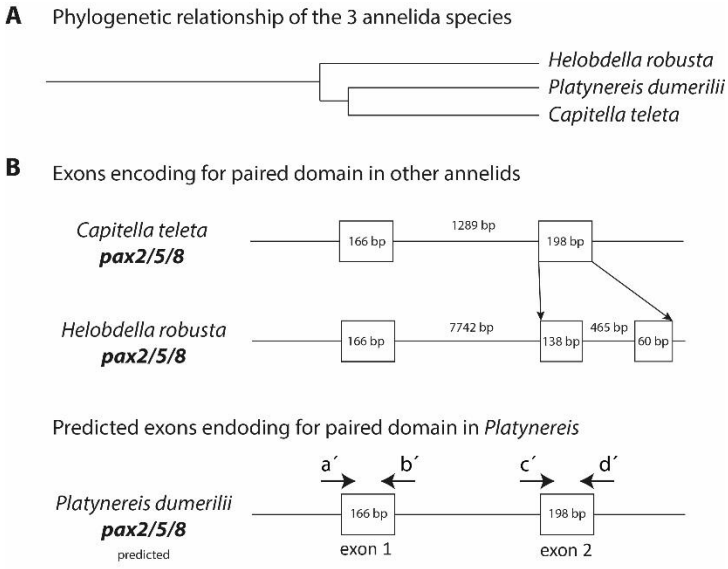

Fig. 17: Gene organisation analysis and genotpying primers design scheme.
A: Phylogenetic relationship of the 3 annelida species used for the genomic sequence comparison. B: Upper panel: *pax2/5/8* gene organisation in the annelid *Capitella teleta* and *Helobdella robusta*. Bottom panel: Inferred gene organization in *Platynereis* and primers design for the 1st and 2nd exon of *pax2/5/8* paired domain.
a´-d´ = genotyping primers. It was confirmed that *Platynereis* has the same gene organisation as *Capitella*, a closer relative than *Helobdella*.

Predicted exon-intron organization of the *Platynereis pax2/5/8* was confirmed by sequencing of target regions, affirming the assumption of the same gene architecture like in the *Capitella teleta.* Gene organisation analysis was performed also for the *Platynereis prox1*, confirming the same gene architecture like in *Capitella teleta* as well.

Platynereis is a relatively new laboratory animal and it is not normally preserved as an inbred line. Therefore, it is necessary to avoid targeting of tools like CRISPR/Cas9 system against highly polymorphic regions, to prevent failure of the mutagenesis tool due to targeting of different sequence from the one used in the original experimental design. Presence of single nucleotide polymorphisms (SNPs) "hot spots" was determined by the alignment of the *Platynereis pax2/5/8* mRNA (cDNA) record from NCBI (accession number KC109637.1) with the *pax2/5/8* sequences taken from 8 individuals from the laboratory breeding. We detected three SNPs hotspots present (Fig. 18).

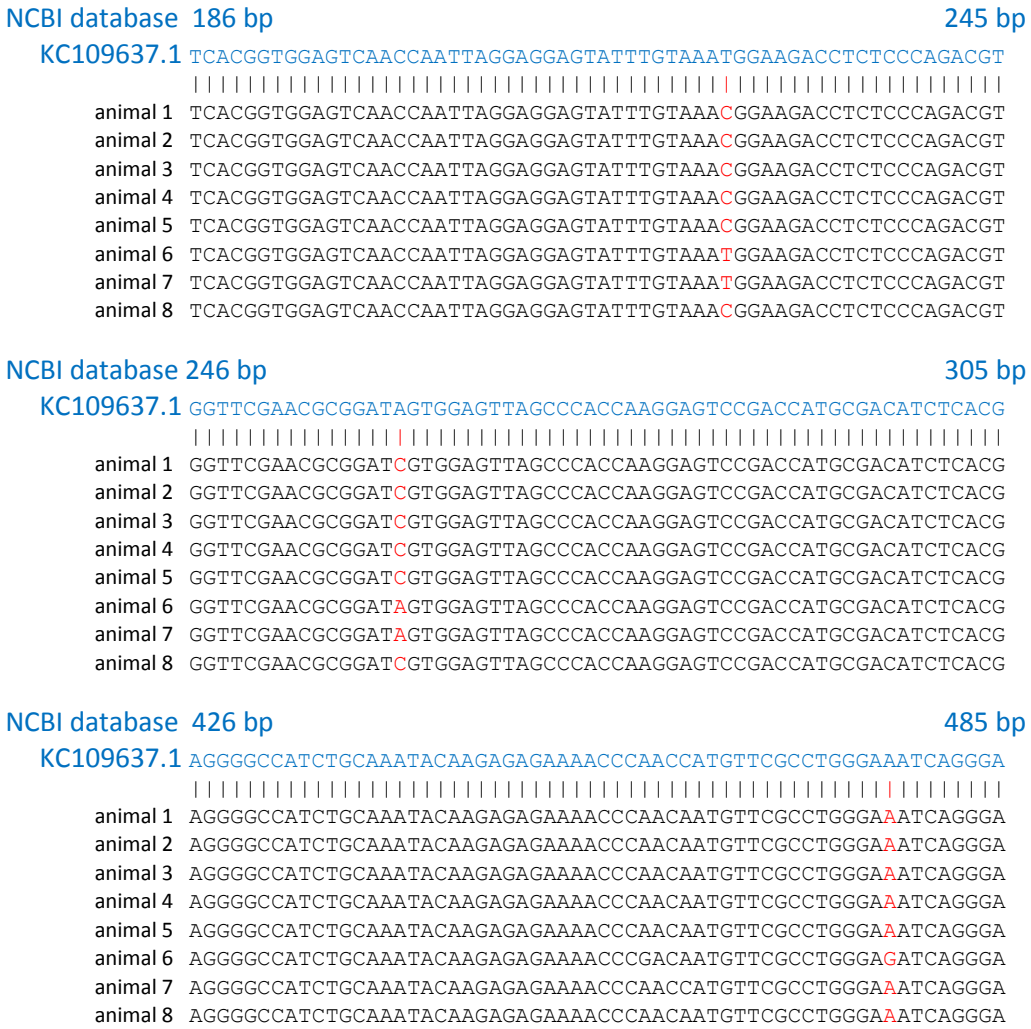

Fig. 18: Alignment of the *Platynereis pax2/5/8* mRNA (cDNA) record from NCBI database (accession number KC109637.1) with the sequences of 1st and 2nd exon of *pax2/5/8* taken from 8 individuals from the laboratory breeding. Primers depicted in fig. 17 B were used for the sequencing (a´- b´ - 1st exon, c´- d´ - 2nd exon). SNPs are highlighted in red, predicted *Platynereis pax2/5/8* sequence marked as blue.

4.3.2 Design of targeted mutagenesis in *Platynereis*

According to the data gained from sequencing of first and second exon regions of *Platynereis pax2/5/8*, target sites for the CRISPR/Cas9 system were chosen in the regions free of SNPs and positioned between primer pairs used for the gene organisation analysis. These primers were used in the following experiments as a genotyping primers. CRISPRs were designed for the target sites 1-4 (Fig. 19). guideRNAs and Cas9 mRNA were co-injected into the one-cell stage *Platynereis* embryos (For more details, see Materials and methods).

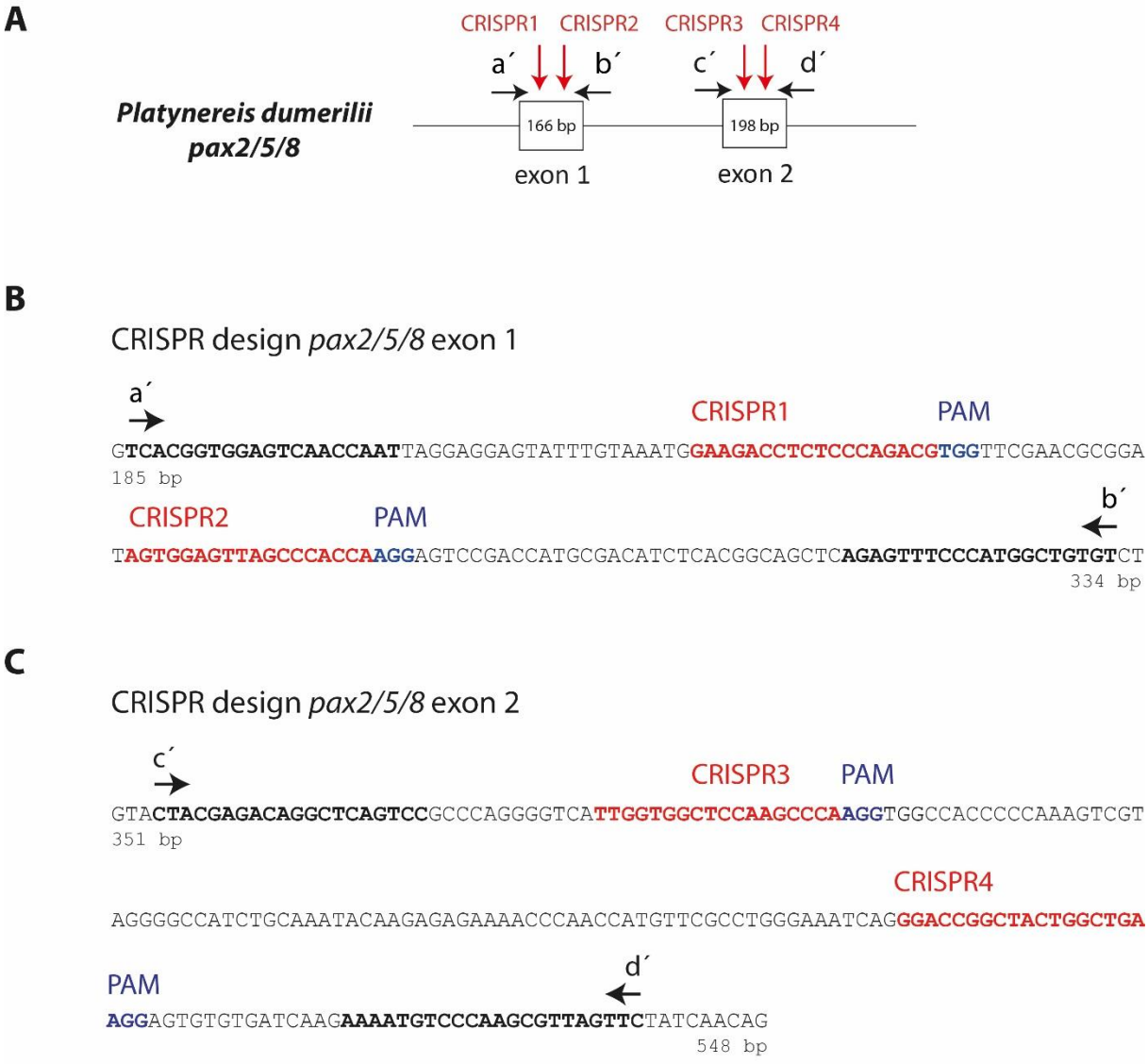

Fig. 19: CRISPR/Cas9 system target sites in the *Platynereis pax2/5/8* gene.
A: Schematic representation of CRISPR design for the *Platynereis pax2/5/8* gene. CRISPR design for B: 1st and C: 2nd exon of predicted *Platynereis pax2/5/8*
CRISPR1-4 = CRISPR/Cas9 system target sites designs highlighted in red, PAM = protospacer-adjacent motif nGG highlighted in blue a´-d´ = genotyping primers.
Platynereis pax2/5/8 mRNA sequence available in NCBI (KC109637.1) was used as a reference.

4.2.3 Validation of the CRISPR/Cas9 system activity in *Platynereis*

To check the efficiency of genome editing by the CRISPR/Cas9 system in *Platynereis pax2/5/8,* we have used different sets of genotyping primers (Fig. 20) Injected larvae (F0 generation) were raised up to 48 hpf, then lysed and subsequently genotyped. Mutations in the first and second exon are visible on the gel as extra bands, indicating deletions, insertions or deletions and insertions present simultaneously in the genome of injected larvae. Deletions of the intron region between the targeted *pax2/5/8* 1st and 2nd exon can be detected as a band presence with primers a´ and d´, since the whole non-mutated region is too long for the PCR amplification and thus no band is detected in the case of wild type *pax2/5/8*. CRISPR/Cas9 ability to provide efficient genome editing in the *Platynereis pax2/5/8* was successfully detected by this genotyping assay (Fig. 20).

Out of the 200 injected embryos during two injection sessions, 65 embryos were lysed after 48 hpf and analysed for the presence of mutations in the *pax2/5/8* exons, 55 embryos were analysed also for excisions and subsequent ligation of regions within the intervening intron. From the animals analysed, 20 were mutated in the 1st exon, 8 in the 2nd exon and 11 animals exhibited excision followed by subsequent ligation. In summary, 25 out of 65 animals tested were detected to be mutants, showing the mutagenesis efficiency to be close to 38.5 %. We have observed diverse mutations in the same embryo due to the mosaic expression. Within the mutated regions, a higher number of deletions compared to insertions was observed.

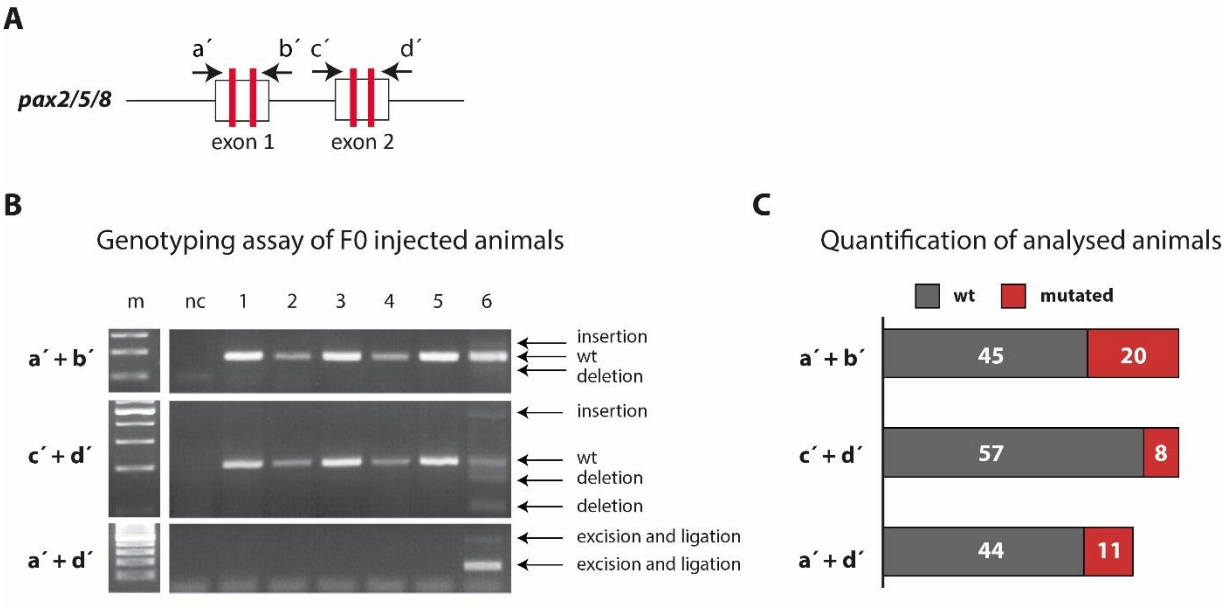

Fig. 20*:* Activity validation of the CRISPR/Cas9 system in *Platynereis* in the F0 injected animals.
A: Scheme of the targeted *pax2/5/8* region, B: Genotyping assay of F0 injected animals, C: Quantification of analysed animals a´-d´ = genotyping primers, m = marker, nc = negative control, 1-5 = wt animals without mutations detected, 6 = mutant animal, multiple deletions and insertions detected within the *pax2/5/8* 1st and 2nd exon, accompanied by excisions and subsequent ligation of regions within the intron in between.

T7-Endonuclease assay was performed to detect the presence of mutations at a single nucleotide level in embryos lysed 48 hpf after the injection. Results showed ability of the CRISPR/Cas9 system to introduce these mutations in the *Platynereis pax2/5/8* and confirmed the mutagenesis efficiency within the targeted loci (Fig. 21). Out of the 200 injected embryos in two injection sessions, 25 embryos were lysed after 48 hpf and analysed for the presence of mutations at the single nucleotide in the *pax2/5/8* 1st and 2nd exon. Mixed samples out of 5 lysed animals were used for each reaction.

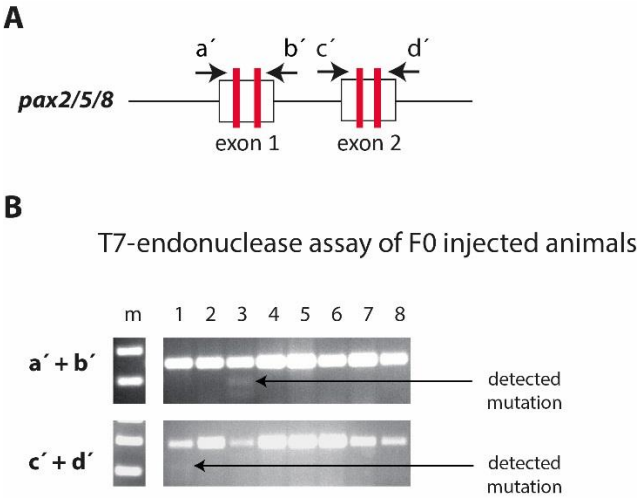

Fig. 21: Activity validation of the CRISPR/Cas9 system in *Platynereis* in the F0 injected animals - T7-endonuclease assay.
A: Scheme of the targeted *pax2/5/8* region, B: T7-endonuclease assay of F0 injected animals
a´- d´ = genotyping primers, m = marker, lane 3 = single nucleotide mutation detected in the *pax2/5/8* 1st exon, lane 1 = single nucleotide mutation detected in the *pax2/5/8* 2nd exon; 2, 4, 5 = F0 animals without single nucleotide mutation detected, lanes 6-8 = control wt animals

For the establishment of the *Platynereis pax2/5/8* knockout line, mutations were successfully introduced into the F0 generation. Currently, F0 animals out of 25 injection sessions are being raised until adulthood, waiting for the founder animals transmitting the CRISPR/Cas9 induced mutation to generate F1 progeny.

5. DISCUSSION

We provide here preliminary analysis of *the Platynereis pax6* mutant line, showing that a single *Platynereis pax6* wild-type allele is sufficient for animal survival. Although heterozygote mutants are viable and able to reproduce, homozygote mutation is lethal and *Platynereis pax6* homozygote mutants die during first weeks of age. Despite the declared synchrony of the *Platynereis* larval development in the literature (Fischer et al. 2010), we have observed substantial variability between individual larvae. However, by comparison of large number of embryos, we show that *otx, six3, pax2/5/8* and *r-opsin*1 genes are not regulated by the *pax6* in *Platynereis* or their regulation is only minimal.

Multiple interaction of *pax6* with *otx, six3, pax2/5/8* and *r-opsin* homologues during the eye development was observed for various organisms in contrast to our data regarding *Platynereis*. Induction of *Otx2* expression triggered by the induced expression of *Pax6* during the ectopic eye formation was detected in *Xenopus* (Chow et al. 1999), similarly positive regulation of *toy* by the *otd* was observed in *Drosophila* embryonic eye (Blanco et al. 2010). In the case of *six3*, *Drosophila* homologue *optix* is regulated by the *ey* (Ostrin 2006). Interactions between *Pax6* and *Six3* were also observed in mouse (Goudreau et al. 2002) or medaka fish (Carl et al. 2002), where they crossregulate each other. Crossregulation was found also in the case of *Pax6* and *Pax2* in mouse, where the PAX6 reciprocally inhibits of *Pax2* promoter and conversely, PAX2 provides inhibition of the *Pax6* (Schwarz et al. 2000). The role of *Pax* genes as regulators of the urbilaterian opsin and its subsequent preservation in the regulation of the r-opsins lineage was predicted by previous research of our lab (Vopalensky 2009).

We did not detect any significant change in the expression patterns of *otx, six3, pax2/5/8* and *r-opsin1* genes between wt animals and *pax6* mutants. This could be attributed to a putative gene redundancy, e.g. in the case another pax6 gene is present, providing functional rescue of the *pax6* knockout. Another case would be that *Platynereis* is not using *pax6* for the regulation of examined genes during the investigated developmental stages in this study. To investigate the first scenario, genome sequencing could be implemented to rule out the presence of another regulating *pax* gene.

Loss of function of *pax6* in *Platynereis* could also lead to loss of function of a certain components of vision, including defects within the brain and neurons, resulting in the possible loss of a larval phototactic behaviour. Since the progeny can be obtained only after the *Platynereis pax6* knockout heterozygote crossing, employment of the behavioral test to sort the affected non-phototactic larvae (probable *pax6* mutants) compared to the normal phototactic ones can be used for the separation of mutated individuals. Subsequently, application of the RNA-seq method on the sorted mutants can provide further analysis of certain components of vision defects caused by the *pax6* knockout. Utilization of two tests during different developmental stages of *Platynereis* can

also give insight into the implication of *pax6* in the regulation of *r-opsin* in larval versus adult eyes. Since only larval eyes are present during the first days of the *Platynereis* life and diminish later, it is possible to implement behavioral tests investigating larval and adult eyes separately.

We have compared two approaches of observing gene expression patterns in *Platynereis* – bright light microscopy and confocal microscopy 3D reconstruction. Due to the considerable variability between individuals in a single batch of *Platynereis* embryos as mentioned above, it is necessary to perform the WMISH procedure on large number of animals to obtain a clear result of expression pattern analysis. In the number of available embryos for comparison, then it is difficult to distinguish pattern differences caused by mutation and natural differences between individuals or differences caused by breeding conditions. Blank animals without the signal are also present within one batch on which WMISH is performed so it is necessary to carefully distinguish between an actual loss of pattern due to the mutation or if it is because of error during WMISH hybridization procedure. Moreover, dead animals may also hamper WMISH pattern readout. In other cases, the reason of blank embryos could mainly be the exclusion of some of the WMISH steps by individual embryo - due to its small size, and subsequently non-functional WMISH hybridization method. Due to the large number of embryos required for a solid result, initial comparison of embryos using light microscopy emerged to be better than more time consuming confocal microscopy. Another reason for faster embryo check light microscopy is the fact that quality of genotyping results declines with longer time after the WMISH hybridization procedure, so it is better to compare and subsequently genotype embryos as close to the WMISH as is possible.

To shed light on the *pax6* gene regulatory network underlying the *Platynereis* eye development, it will be necessary to study other genes that may be involved in this process. Based on the utilization of known data from other related model organisms, putative participants within the Lopchotrochozoa lineage may be deduced.

Since we did not detect any change in the *r-opsin1* expression pattern in the *Platynereis pax6* knockout animals, we selected *pax2/5/8* as another potential gene involved in the *Platynereis r-opsin1* regulation. We have analysed expression pattern of *pax2/5/8* in *Platynereis* for the first time and we further compared utilisation of the bright field imaging using light microscopy and 3D reconstruction using confocal microscopy for the investigation of the WMISH pattern in *Platynereis*. Confocal microscopy 3D imaging using increasing rising compensation of the laser, reveals to be the best option for the detailed expression pattern description. Expression of *pax2/5/8* in the *Platynereis* larval and adult eyes was successfully confirmed.

To elucidate the function of the *Platynereis pax2/5/8*, we decided to generate a stable knockout line. We prepared a functional tool for mutagenesis of the *Platynereis pax2/5/8* using the CRISPR/Cas9 system (Jinek et al. 2012; Jao et al. 2013) and demonstrated its functionality *in-vivo*.

Exon-intron organization of the *Platynereis pax2/5/8* gene was predicted by *in-silico* comparison of *pax2/5/8* genes from related species and the analysis was afterwards confirmed in *Platynereis in-vivo*. This analysis revealed common positions of SNPs in *pax2/5/8* in the *Platynereis* breeding culture. Due to its critical role in binding to DNA, paired domain of *pax2/5/8* was selected as a target region for generating mutations using the CRISPR/Cas9 system. Targeting the first two exons of the paired domain should provide complete loss of Pax2/5/8 protein function, however, even a single nucleotide mutation within the paired domain is assumed result in loss-of-function genomic change (Kozmik et al. 1997). Insertions containing more amino acids or deletions, similarly as frameshift mutations lead to the disruption of the protein structure and non-functional translational products. Loss-of-function could be also a consequence of a frameshift mutation leading to a change in reading frame, which can result into the termination of translation due to the premature formation of a stop codon. Even an in-frame single nucleotide change leading to the change of one of the crucial amino acids within the paired domain is considered to promote function malformation.

Functionality of the CRISPR/Cas9 tool was confirmed on the 48 hpf old *Platynereis* embryos, revealing mutations of variable size, ranging from single nucleotide mutations up to deletions and insertions large enough to be easily detectable by the used genotyping assay. Mutagenesis seems to be more effective in the case of the first exon of *pax2/5/8*, which could be caused by better CRISPR/Cas9 system activity in the targeted sites of the first exon as compared to the second one. Another possible reason could be the presence of a SNP within the targeted region and thus disparity between the sequence used originally for the tool design and the actual sequences of various individuals. It is possible that SNP in the second has a low occurrence frequency, thus was not previously detected during sequencing analysis of the target regions. Interesting could be also comparison of usage of Cas9 protein instead of Cas9 mRNA to provide immediate source of the cleaving enzyme, which could also affect the genome editing realization (Wang et al. 2015; Gagnon et al. 2014; Sung et al. 2014).

F0 generation of injected animals was analysed using the genotyping assay. Multiple bands visible on electrophoresis gels are caused due to the persistence of CRISPR/Cas9 cutting activity until the target site is destroyed, however, occurring differently in each cell of dividing embryo resulting into the mosaic expression (Hsu et al. 2014; Yen et al. 2014). T7-endonuclease assay was performed as an additional step of the analysis of F0 injected animals, thus analysing mutations at the single nucleotide level. Distinction of SNPs caused by the CRISPR/Cas9 system activity in the targeted regions and naturally occurring SNPs in the *Platynereis* genome is feasible due to the higher number of naturally occurring SNPs as compared to the SNPs caused by the CRISPR/Cas9. In other words, mismatches caused by the naturally presented SNPs occur within the same sites in higher number of analysed animals compared to the SNPs caused by the CRISPR/Cas9.

To establish a stable mutant line, it is necessary to continue with analysis in following generations following the F0 injected animals. PCR genotyping is performed on the F0 animal piece of tail, previously cut and lysed, however, due to the mosaic expression of the mutated gene, mutated cells may not be present in the tail and thus providing false negative results. Following cross of F0 injected animals against wild-type animals is necessary to identify the carriers of the mutant allele within the germline. This is needed to obtain stable transmitted mutation across next generations and to avoid the possible off-target mutagenesis.

6. CONCLUSION

Molecular regulation of the early eye development of *Platynereis dumerilii* was investigated during this thesis. We have successfully analysed expression of the *pax6* and *pax2/5/8* in the wild-type *Platynereis dumerilii*, showing the expression patterns of these genes in 24, 48 and 72 hpf old embryos. Furthermore, we have compared two approaches of observing the gene expression pattern – bright light microscopy and confocal microscopy, and show that both methods are applicable. To investigate the role of *pax6* and *pax2/5/8* in eye during the *Platynereis* development, analysis of an existing *pax6* knockout line was performed along with implementation of the CRISPR/Cas9 system to generate a *pax2/5/8* knockout line.

During the analysis of the *pax6* knockout line, it was show that just one *pax6* wild-type allele is sufficient for the *Platynereis* survival and ability to reproduce. Data collected during the analysis of the *pax6* knockout line are further suggesting that *pax2/5/8, otx, six3* and *r-ops1* are not regulated by *pax6* in *Platynereis*. Interestingly, *pax6* is not regulating *r-ops1* in the *Platynereis* adult eyes, contrary to the conserved role of the *pax6* in the regulation of eye development across many animal phyla.

pax2/5/8 was selected as another *pax* gene, which may be involved in the r-opsin regulation in the *Platynereis* eyes. To investigate the role of the *pax2/5/8* during the *Platynereis* development, CRISPR/Cas9 was successfully implemented into the *Platynereis* to provide the *pax2/5/8* knockout. The efficiency of the CRISPR/Cas9 was tested and verified as a functional tool able to generate mutations detectable by genotyping in *Platynereis in-vivo*.

We therefore conclude that although *Platynereis* has started to be used relatively recently, it is a suitable model system for the evolutionary developmental biology studies. Utilization of new emerging molecular biology methods will provide approach for the broad research within the topic of the eye development.

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